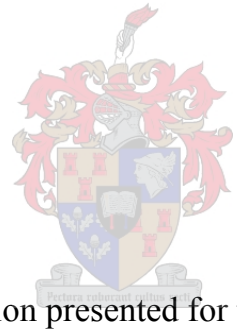
The evaluation of the impact of microclimatic factors on grapevine berries in a vineyard setting through molecular profiling

by

Kari du Plessis



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Declaration

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Summary

Grape composition is considered to be the result of the grapevine genotype, the environmental factors the grapes are exposed to and the management practices implemented during their development. However, elucidating how each of these components contributes to the outcome is notoriously difficult under field conditions due to the myriad confounding variables that grapes are influenced by. One of the viticultural management practices frequently implemented in the vineyard is the removal of leaves in the berry bunch zone in order to alter the microclimate of the developing grapes with various potentially advantageous outcomes. However, this common viticultural practice of leaf removal very rarely affects levels of light without elevating bunch temperatures as well. Moreover, definitive links between this treatment and the underlying grape molecular responses are currently lacking, particularly in cause and effect relationships.

Utilizing a highly characterized Sauvignon Blanc vineyard, a leaf removal treatment was implemented according to a field-omics experimental approach, in which it was established that light exposure to the developing grapes was the predominant factor modulated by the treatment. A preceding study characterized the physical growth parameters of the developing grapes and targeted specific metabolites in order to determine how elevated light would affect grape development. The results revealed that the growth and development of the grapes were not affected by the treatment, but that specific secondary metabolites with photoprotective abilities were elevated. These results showed that the grapes acclimated to the elevated light exposure, providing the possibility to study the molecular mechanisms associated with this acclimated state in the berries.

The aim of this study was therefore to explore the transcriptional responses of the developing grape berries to elevated light exposure to understand how primary metabolism and growth was maintained despite the implementation of stress mitigation strategies. The approach taken to study this transcriptional response involved RNA sequencing (RNASeq) analysis in order to generate a transcriptional snapshot of all the genes expressed in control and light exposed grapes sampled at four developmental stages throughout berry development. This analysis revealed that the green grapes implemented several photoprotective mechanisms simultaneously. Some of these mechanisms involved non-photochemical quenching and the rapid turnover of the proteins of the photosynthetic machinery, much like other foliar photosynthetic tissues, despite the profound differences in photosynthesis dynamics between these tissue types. Additionally, the genes associated with the synthesis of flavonoid compounds were significantly upregulated and these findings were further corroborated by the accumulation of high levels of flavonols that are known to have both light absorbing and antioxidant abilities. In combination, through these photoprotective mechanisms, as well as the synthesis of higher levels of carotenoids in green berries and subsequent apocarotenoids in ripe berries these grapes achieved a state of acclimation. Furthermore, the catabolism of amino acids provided energy precursors and substrates towards the redistribution of energy that contributed to the maintenance of these energetically costly stress mitigation mechanisms. To this end, green, photosynthesizing grapes maintain growth and development at all costs to protect the development and maturation of the grape seed. Therefore, when the berries achieved ripeness, the photoprotective mechanisms associated with photosynthesis had ceased and the upregulation of apocarotenoids and flavonols were no longer effectively mitigating the light stress.

A subsequent investigation explored the role that grapevine heat shock factor (Hsf) genes may have played in achieving this acclimated state. The consistent upregulation of three grapevine *Hsfs* was established and for one of these genes, *VviHsfA7a*, a unique putative role in photoprotection under elevated light was identified. Furthermore, by utilizing these results, the first putative working model of the expression and regulation of the *Hsfs* in grapevine berries were proposed.

This study further identified two groups of putative developmental stage-specific molecular biomarkers in grape berries. The first group of genes contributed to the current understanding of the underlying molecular mechanisms associated with the coordinated progression of berry development, whereas the other group of genes represented putative light-responsive molecular biomarkers that are developmentally regulated under non-stressed conditions, but that become significantly upregulated by light stress.

Further investigation into the effect that the elevated light exposure may have had on the pathways associated with the synthesis of Sauvignon Blanc impact odorants was conducted. These findings provided insights into how leaf removal and elevated light exposure may lower green aroma characteristics in wine by modulating berry metabolism on a molecular level.

Taken together, the findings presented in this study provided definitive insights into how light exposure effects grape berry development on a molecular level and the mechanisms that these berries implement in order to ameliorate the potentially harmful affects of light stress. This study further contributed by putting forward the first *de novo* assembled transcriptome for the Sauvignon Blanc grapevine genotype that can be utilized in future studies in order to draw more conclusive links between genotypic and/or treatment specific expression in grapevine.

Opsomming

Druif samestelling word bepaal deur die wingerd se genotipe, die omgewigsfaktore waaraan die druiwe blootgestel word en die bestuurspraktyke toegepas tydens die druif se ontwikkeling. Om uit te vind hoe elkeen van hierdie komponte spesifiek bydra tot die finale druifuitkoms is dikwels moeilik onder veld-toestande weens die magdom interafhanklike veranderlikes wat die druiwe beïnvloed. Een van die bestuursgebruike wat algemeen gebruik word, en wat verskeie moontlike voordelige nagevolge kan hê, is die verwydering van blare in die druif trossone ten einde die mikroklimaat van die ontwikkelende druiwe te verander. Hierdie algemene blaar verwyderingstegniek beïnvloed nie net die vlakke van lig blootstelling nie, maar laat ook meestal trostemperature styg. Duidelike skakels tussen hierdie behandeling en die onderliggende molekulêre reaksies van die druiwe ontbreek nog, veral as gesoek word na oorsaak-en-effek verhoudings. In ‘n vorige studie, deur gebruik te maak van ‘n hoogs-gekarakteriseerde Sauvignon Blanc wingerd, kon bevestig word dat lig bloostelling aan die druiwe die hoof variërende faktor was in ‘n “field-omics” blaarverwyderingsbehandeling. Die fisiese groei parameters van die ontwikkelende druiwe en geteikende metaboliete is ook reeds gekarakteriseer om te bepaal hoe hoër vlakke van ligblootstelling die druiwe se ontwikkeling sou beïnvloed. Die resultate het onthul dat die algemene groei en ontwikkeling van die druiwe nie beïnvloed was deur die behandeling nie, maar dat die vlakke van spesifieke sekondêre metaboliete wat rolle vervul in fotobeskerming, hoër was. Hierdie resultate het getoon dat die druiwe aangepas het tot die hoër vlakke van ligblootstelling en dit het die bestudering van die molekulêre meganismes onderliggend aan hierdie aangepaste toestand moontlik gemaak.

Die doel van hierdie studie was dus om die transkripsionele reaksies van die ontwikkelende druiwe tot hoër ligvlakke te verken om te verstaan hoe primêre metabolisme en groei gehandhaaf kon word ongeag die feit dat die druifkorrels besig was met stres verminderingsstrategië. Die aanslag van die studie was om hierdie transkripsionele reaksiese te bestudeer met RNA sekwensiëringanalise (RNASeq) sodat ‘n transkripsionele oorsig van al die gene en hul uitdrukking in kontrole- en lig-blootgestelde druiwe gegenereer kon word tydens vier spesifieke druif onwikkelingsstadia. Die analise het onthul dat die groen druiwe verskeie fotobeskermingsmeganismes gelyktydig geïmplementeer het. Sommige van hierdie meganismes behels nie-fotochemiese blussing en vinnige omskakeling van die proteïene wat deel vorm van die fotosintetiese masjienerie, soortgelyk aan ander blaaragtige fotosinterende weefsels, ongeag die definitiewe verskille in fotosintetiese dinamika tussen hierdie weefseltipes. Verder was die gene betrokke by die sintese van flavonoïedverbindings beduidend opgereguleer. Hierdie bevindinge was verder ondersteun deur die versameling van hoër vlakke van flavonole wat bekend is vir beide hul lig-absorberende- en antioksidantvermoëns. Deur middel van hierdie fotobeskermingsmeganismes, asook die sintese van hoër vlakke van karotenoïede in groen druiwe; en die gevolglike vorming van apokarotenoïede in ryp druiwe, is ‘n ligstresaangepasing behaal. Verder het die katabolisme van aminosure energie voorgangers en substrate voorsien vir die herverspreiding van energie hulpbronne wat bygedra het tot die handhawing van hierdie stres-verminderingsmeganismes. Dit blyk dus asof die fotosinterende druiwe groei en ontwikkeling gehandhaaf het ten alle koste om sodoende die ontwikkeling en rypwording van die druif-sade te beskerm. Verder, toe die druiwe rypheid behaal het, is die fotobeskermingsmeganismes geassosieërd met fotosintese gestaak en die opregulering van apokarotenoïede en flavonole was nie meer genoegsaam om die ligstres te onderdruk nie.

‘n Opvolgondersoek het die rol van wingerd hitte-skok faktor (Hsf) gene wat moontlik ‘n rol gespeel het in die aangepaste toestand ondersoek. Die volgehoue opregulasie van drie wingerd *Hsfs* is bevestig en vir een van hierdie gene, *VviHsfA7a*, is ‘n unieke vermeende rol in fotobeskerming onder hoër ligvlakke geïdentifiseer.

Verder, deur die toepassing van hierdie resultate is die eerste vermeende werksmodel van die uitdrukking en regulering van die *Hsfs* in druiwekorrels saamgestel. Hierdie studie het verder ook twee groepe

vermeende ontwikkelingsstadia-spesifieke molekulêre biomerkers in druiwekorrels geïdentifiseer. Die eerste groep gene het bygedra tot die huidige kennis van die onderliggende molekulêre meganismes geassosieër met die gekoördineerde verloop van druifontwikkeling, terwyl die ander groep gene vermeende lig-reaktiewe molekulêre biomerkers aangedui het wat gereguleer word deur ontwikkeling onder nie-stres toestande, maar wat beduidend opgereguleer word as daar ‘n lig stres ervaar word.

Ondersoeke na die effek wat hoër ligblootstelling kon hê op die paaie geassosieër met die sintese van Sauvignon Blanc impakgeure is ook uitgevoer. Hierdie bevindinge het insig verskaf in verband met hoe blaarverwydering en hoër vlakke van ligblootstelling groen geur-eienskappe in wyne kan verlaag deur die modulering van druif-metabolisme op ‘n molekulêre vlak.

In samevatting, die bevindinge van hierdie studie kon definitiewe insigte gee rakende hoe ligblootstelling druifontwikkeling beïnvloed op ‘n molekulêre vlak en die meganismes wat hierdie druiwe implementeer om die moontlik skadelike nagevolge van lig stres te beperk Hierdie studie het verder bydraes gemaak deur die generering van die eerste *de novo* saamgestelde transkriptoom vir die Sauvignon Blanc druifgenotipe wat in toekomstige studies toegepas kan word om meer definitiewe skakels te maak tussen genotipe- en/of behandling-spesifieke geenuitdrukkings in druifstudies.

This dissertation is dedicated to
Leo and my parents

Biographical sketch

Kari du Plessis (née van Rensburg) was born on 26 December 1985 and raised in Strand. She matriculated from Strand High School in 2003 and commenced her studies at the University of Stellenbosch in 2007 where she enrolled for a BSc-degree in Biodiversity and Ecology which she obtained in 2009. She received a BScHons- degree in Wine Biotechnology in 2010 after which she graduated with an MSc-degree (Cum Laude) in Wine Biotechnology at the Institute for Wine Biotechnology at Stellenbosch University.

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- “Die Span”

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- Leo, my husband, for everything. Thank you for patiently kick-starting the life we dreamed of while I was busy pursuing my own professional challenges. For that I will be eternally grateful.

Preface

This dissertation is presented as a compilation of seven chapters. Each chapter is introduced separately and is written according to the style of the journal, Frontiers in Plant Science, in which Chapter 3 was published.

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**
Grapevine berry development and light exposure: A review

Chapter 3 **Research results**
The transcriptional responses and metabolic consequences of acclimation to elevated light exposure to elevated light.

Chapter 4 **Research results**
The identification and evaluation of developmental and light responsive molecular biomarkers in Sauvignon Blanc grapes.

Chapter 5 **Research results**
Heat shock factor, HspA7a, may be involved in grape berry acclimation to elevated light exposure.

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Chapter 7 **General discussion and conclusions**

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Chapter 1

General Introduction and Project Aims

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General Introduction and Project Aims

1.1 Introduction

Plants show tremendous adaptability to adverse environmental conditions in order to maintain efficient growth and reproduction regardless of numerous abiotic stresses. Abiotic stress responses have been extensively studied in model plant systems and are increasingly also being studied in crop plants to ultimately allow rational improvements to cultivation and management practices.

Grapevine is currently considered to be the most economically important fruit crop worldwide and is being cultivated in 72 countries according to the Food and Agriculture Organization (United Nations, 2015). There is, however, an unpredictability associated with the cultivation of grapes in a field setting that could largely be attributed to the interaction between the specific grape genotype being cultivated and the environmental factors that the developing grapes are exposed to. These environmental conditions that influence grape development can be further exacerbated by specific viticultural (management) treatments. Accordingly, grapes are considered to be the result of the interaction between the specific grape genotype (G - cultivar), the environment (E) they are exposed to and the viticultural management (M) practices implemented throughout the development of the grapes in the so-called GxExM interaction.

"Terroir" is the term frequently used to collectively describe the outcome of this interaction between the vines, their environment and the effect of human intervention in a specific geographic location (reviewed in Fabres et al., 2017). The use of a simple term to describe such a highly complex interaction remains problematic since it is still shrouded in uncertainty regarding the multitude confounding variables potentially impacting on the ultimate outcome/product. Another term frequently used to describe the variability between harvest seasons on product quantity and quality parameters is the "vintage effect". This description further highlights the complex interactions within a vineyard setting, but does still not provide for conclusive links between the specific variable and the ultimate outcome. Grape research is benefitting currently from efforts to systematically describe and monitor the variable factors that can impact on growth, development and product quantity/quality. Technological advances are paramount to allow for closer investigation into these complex GxExM interactions.

In the research of grapevines, several technological advances have lead to a clearer understanding of the highly programmed progression associated with the developmental patterns in grapevine since the sequencing of the grapevine genome (Velasco et al., 2007; Jaillon et al., 2007) that in turn lead to the study

of whole grape transcriptomes through Microarray and RNA sequencing (RNASeq). Whole genome comparisons have revolutionized our understanding of the genetic relatedness of the commercial cultivars we work with (Myles et al, 2011) and has sparked renewed efforts to study the genetic bases of disease and environmental vulnerabilities (Blanco-Ulate et al., 2015; Carbonell-Bejerano et al., 2013; Dal Santo et al., 2016; Ghan et al., 2015; Martinez-Luscher et al., 2014; Matus et al., 2016; Reshef et al., 2017; Rienth et al., 2014; Savoi et al., 2016; Sun et al., 2017; Suzuki et al., 2015; Wu et al., 2014), as well as important traits such as flowering and fruit formation (Rienth et al., 2014; 2016; Luchaire et al., 2017; Royo et al., 2016; Díaz-Riquelme et al., 2014), berry flesh formation (Fernandez et al., 2013) and the molecular analysis of berry color (Kobayashi et al., 2004; Carbonell-Bejanero et al., 2017). Furthermore, advancements in the field of metabolomics and proteomics have allowed for novel research identifying the specific biomarkers associated with each of the stages of grape berry ripening by integrating transcriptomics, metabolomics and proteomics through the development of bioinformatics pipelines (Zamboni et al., 2010). The Grape Gene Expression Atlas (Fasoli et al., 2012) describing the expression patterns of 54 diverse samples dramatically advanced our understanding of the underlying molecular signatures associated with the development of most grapevine tissues and organs. Similarly, an atlas for small RNAs has been established that defined the role of microRNA distribution in grapevine organ identity (Belli-Kullan et al., 2015). These studies, among many others (Costantini et al., 2017; Carbonell-Bejerano et al., 2016; Vondras et al., 2017; Wang et al., 2017 to name only a few recent examples), are contributing to the more predictable cultivation of grapes through clarifying the development of the vines and grapes themselves.

Not only have the grapevine profiling tools advanced, but the technologies available with which to characterize specific environmental conditions have improved dramatically as well. Sensors are now available to more accurately quantify the macro, meso and microclimatic variables of the developing grapes in a field-setting and studies that have employed these techniques have further revealed the complex variable nature of the environmental conditions in a vineyard and its impact on grape development (Anesi et al., 2015; Carbonell-Bejerano et al., 2016; Pinto et al., 2016).

In a vineyard setting, grapevines are not only frequently exposed to abiotic stresses as a result of natural environmental fluctuations, but common agronomical interventions may further induce abiotic stresses. It is therefore with the establishment of the berry developmental features under field conditions that it has become possible to more accurately determine the effect that abiotic stress conditions may have on the outcome of berry composition. It is by implementing a field-omics approach in the planning, execution and integration of several omics disciplines in the field (Alexandersson et al, 2014), that grapevine field experiments could dramatically advance our understanding of the transcriptional and metabolic responses associated with berry abiotic stress exposure. One example of the most recent successful omics integration reported on the effect of water deficit on Merlot grapes in which the authors integrated data generated from RNASeq analysis and metabolite measurements to ultimately propose which molecular mechanisms are involved in the berries' response to water deficit in the field (Savoi et al., 2016). Among the many

viticultural interventions that will inevitably influence grape berry development is the common practice of leaf removal in the berry bunch zone. This practice is associated with several advantages that may include aiding in pest management by lowering canopy humidity and therefore fungal infection (Chellemi and Marois, 1992; English et al., 1989), providing a balance in crop load (Palliotti et al., 2012) and making the fruit more accessible during harvest. Additionally, the quality and sensory attributes of grapes grown on defoliated vines are known to be different from shaded grapes. These altered quality parameters include balanced pH levels in grape juices (Hunter and Visser, 1990; De Toda et al., 2013) and a reduction of green aroma characteristics in the resulting wines (Šuklje et al., 2016). Furthermore, in red wine cultivars, an improvement in color stability is also reported in grapes exposed by leaf removal (Chorti et al., 2010; Lee and Skinkis, 2013). However, despite these reported outcomes, definitive links between this treatment and the underlying molecular responses have not been fully elucidated yet and deserve attention, specifically to provide cause-and-effect validations.

Recently the effect that leaf removal may have on grape development has been evaluated by integrating several omics technologies. Although it was reported that the applied treatment affected both light and temperature to various degrees, the authors did not conclude on the unique effects that either light or temperature may have had on the metabolic outcome of the grapes (Pastore et al., 2013; Sun et al., 2017). This is important since it was previously established that light and temperature differentially affects the metabolic pathways frequently associated with increased exposure to grapes (Azuma et al., 2012). The effects of elevated whole-plant temperature on grape development was skillfully integrated in a greenhouse experiment (Pastore et al., 2017) and has shown that, as with many stress response studies, that processes of acclimation is key and that phenotypic plasticity is orchestrated on several integrated levels.

1.2 The aims and objectives of this study

This study had the benefit of an existing highly characterized (model) vineyard where the field-omics principles were implemented in the design of the experiments. The model vineyard was in the Elgin region (Western Cape, South Africa), a high altitude, cool climate area, where predictable sea breezes have a modulating effect. It was a Sauvignon Blanc vineyard where the experimental layout created two distinct microclimates in the bunch zone by implementing a leaf removal treatment in the morning side of the canopy to established an exposed bunch zone versus a non-treated control where the bunches were covered by a leaf layer. Prior to this study, the meso- and microclimatic conditions of the vineyard and the treated panels were monitored to evaluate the outcomes of the treatments in terms of microclimatic features altered. At the onset of this study, the main variable of the leaf removal treatment was confirmed to be light exposure and not temperature, since data from a range of sensors showed that at the specific location and in this vineyard, the light incidence on the berries were significantly modulated, whereas berry temperatures were not significantly affected. Targeted metabolite analyses of the berries throughout development and ripening were implemented to study the metabolic reaction to the treatment. These included an analysis of

the sugars and organic acids of the grapes throughout development and ripening. Since the main variable was confirmed to be light, chlorophyll and carotenoid pigments were also measured to evaluate possible impacts of the increased light exposure. The data showed that the ripening pattern and the sugars and organic acid levels were largely similar in the exposed and control grapes, but that specific carotenoid pigments, specifically the photoprotective xanthophylls, as well as certain ratio's of the pigments were dramatically affected. It was evident that the berries adapted to the high light and the low light environments in unique ways. These interesting results prompted a working hypothesis that the leaf removal treatment and the level of exposure lead to an acclimated state in the berries that will affect the metabolism of the berries throughout development and ripening. Several experiments were designed to test this working hypothesis and one of these was the start of the current study, where the focus fell on the use of transcriptomic analyses as a non-targeted approach to profile the reaction of the grapes and to guide further, more targeted analyses.

The predominant aim of this study was therefore to evaluate the effect of elevated light as the result of a leaf removal treatment to grape berry development through whole-transcriptomic analysis.

The specific aims, the approaches taken and the specific thesis sections in which each aim is addressed are summarized as follows:

1. **Planning and performing whole-transcriptome analysis of Sauvignon Blanc grape berries sampled at four phenological stages throughout their development in a highly characterized vineyard where light exposure was the predominant effect of a leaf removal treatment.**

2. **The characterization of the global transcriptional signatures associated with the development of these grapes and the effect that the treatment may have had on these developmental profiles.**

3. **Utilizing the generated transcriptional data to identify the molecular mechanisms most significantly affected by the increased light in order to target downstream analyses to support the transcriptomic results.**

4. **Integrate the transcriptome and targeted metabolite data to generate a holistic overview of how increased light exposure affects the berry transcriptome and metabolism and to potentially link these responses to Sauvginon Blanc wine quality impact factors.**

The outputs presented in this thesis addressed these aims in the following order:

- Chapter 3 (addresses aim 1, 2, 3 and 4):

    - Utilizing the initial metabolite data generated from the grapes sampled from this vineyard in order to select a subset of biological replicates for RNASeq analysis with the lowest statistical variability among the grapes sampled from light exposed and control grapes sampled at four phenological stages. The selection strategy was presented as a poster at the 2014 Macrowine Conference (Addendum A of Chapter 3).

    - Performing whole-transcriptome RNASeq analysis on control and light exposed Sauvignon Blanc grapes sampled at four phenological stages throughout their development.

    - Targeting the genes involved in carotenoid/norisoprenoid metabolism in order to support the metabolite data generated before the commencement of this PhD project where the main impacts of the treatments were validated to form part of a publication (Young et al., 2016) presented in Addendum B of Chapter 3.

    - Characterizing the global transcriptional responses of developing grape berries exposed to elevated light, utilizing the results towards targeting downstream analyses and to integrate the transcriptomic and metabolomic data for the purpose of creating a holistic overview of how grape berries respond to elevated light exposure.

    - Chapter 3 which has since been published as Du Plessis et al., (2017), as well as supplementary materials that were not part of the publication, are presented in Addendum C to Chapter 3.

- Chapter 4 (addresses aim 2):

    - Based on results reported in Chapter 3, the molecular stage-specific berry developmental biomarkers representing the green and the ripening stages of berry development were identified and characterized in order to contribute to the establishment of the underlying molecular signatures that represent the well-established progression of berry development regardless of the treatment implemented.

    - The identification of putative light-stress associated molecular biomarkers that are developmentally driven under non-stressed conditions but that are significantly affected by elevated light.

- Supplementary materials, methods and results are also presented in Addendum A of Chapter 4.

- Chapter 5 (addresses aim 3 and 4):

  - Based on results generated in Chapter 3, the expression of Heat Shock Factor (Hsf) encoding genes were targeted in order to establish their potential role in grape berry responses to elevated light exposure.

  - The generation of the first working model for the expression and regulation of Hsf encoding genes in grapes.

  - Supplementary materials, methods and results are presented in Addendums A, B and C of Chapter 5.

- Chapter 6 (addresses aim 4):

  - In order to expand on the results generated in Chapter 3 and Addendum B of Chapter 3, transcriptional data generated was utilized to evaluate the metabolic pathways associated with the synthesis of the Sauvignon Blanc impact odorants that include methoxypyrazines, green leaf volatiles and volatile thiol precursors in the grapes. The transcriptional data was supported by data previously generated for the concentrations of amino acids (Chapter 3) and green leaf volatiles (Addendum B of Chapter 3).

  - Benefitting from the broader program in this model vineyard where experimental wines were also made from the exposed and control grapes by co-workers, previously reported data regarding three volatile thiols measured in the wines could be used to also relate the accumulation of these compounds in the final product to the transcription of the genes associated with precursor synthesis in the grapes in the exposed and control treatments.

  - Supplementary materials, methods and results not presented in Chapter 6 are presented in Addendum A of Chapter 6.

The thesis also contains a literature review on grapevine berry development and light exposure in Chapter 2 and is concluded with a general concluding discussion in Chapter 7.

My supervisor, Prof MA Vivier and co-supervisor, Dr PR Young, were responsible for conceptualizing and planning the study and their critical evaluation of the project and results generated was continuously ongoing. Dr PR Young implemented and maintained the viticultural treatments and was responsible for

berry sampling. I was responsible for the planning and execution of the RNA extractions and purifications with the help of Dr J Belli-Kullan for the purpose of RNASeq analysis. The RNASeq analysis and raw data processing was outsourced to the Department of Biotechnology at the University of Verona (Italy). I performed all subsequent bioinformatic data analyses and the biological interpretation of the results. Dr H Eyéghé-Bickong performed HPLC analysis for the determination of amino acid and phenolic compound concentrations and I performed the statistical analysis and interpretation of the results. The lipophilic antioxidant capacity of the grapes was determined by Mr F. Rautenbach at the Oxidative Stress Research Centre at the Cape Peninsula University of Technology. The interpretation of all the results and completion of the written work towards the compilation of this thesis was performed by myself, under the guidance of my supervisors, Prof MA Vivier and Dr PR Young.

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Chapter 2

Literature Review

Grapevine berry development and light exposure:

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2.1 Introduction

Plants sustain themselves and almost all life on Earth through their ability to harvest energy from atmospheric sunlight through photosynthesis. It has, however, been established that the rate of photosynthesis can readily switch from high levels to very low levels in response to the quality and quantity of light that the plant is exposed to. This environmentally induced, coordinated mechanisms with which plants respond to light is collectively described as light acclimation. The light fluctuations that plants may perceive could either be under natural, predictable conditions such as diurnal or seasonal changes, or due to unpredictable conditions caused, for example, by intermittent cloud cover or dappled shading inside the plant canopy understory. Furthermore, in the case of commercially important food crops, agronomical interventions such as pruning or training of canopies may further induce significant fluctuations in plant exposure to light.

Light acclimation involves adjustments of not only chloroplastic activities, but various non-chloroplastic processes as well. These processes (Figure 2.1) have been recently extensively reviewed in a special issue of the Philosophical Transactions of the Royal Society of London, as summarized by Spetea et al. (2014).

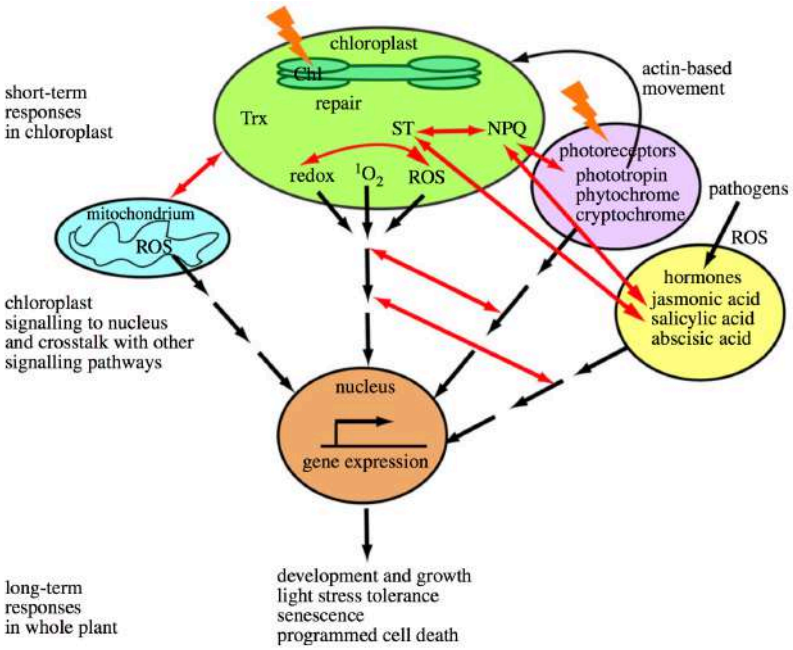

Figure 2.1. An overview of light-induced chloroplast signaling and response mechanisms as presented by Spetea et al., (2014). Chl, chlorophyll; NPQ, non-photochemical quenching; ROS, reactive oxygen species; ST, state transition; Trx, thioredoxin.

Light is inevitably linked to the process of photosynthesis in plants. Aside from leaves as predominant photosynthetic organs, other plant tissues contain functional chloroplasts and therefore are capable of photosynthesis (for review, see Blanke and Lenz, 1989). Some of these photosynthetic organs include stems, tendrils, flowers and green fruits. There are however notable differences in the dynamics of photosynthesis when comparing leaves to non-foliar tissues. In the case of photosynthesizing fruits, photosynthesis decreases as fruit ripen, partially due to the gradual disappearance of stomata or the development of a waxy cuticle that establishes a hypoxic internal environment in the fruit, hereby reducing the photosynthetic activity (Blanke and Leyhe, 1987; Kyzeridou et al., 2015).

In this review the focus will fall on light in interaction with grapevines, specifically on berry growth and metabolism. The European grapevine, *Vitis vinifera*, is currently considered to be the most important fruit crop worldwide, cultivated in as many as 72 countries according to the Food and Agriculture Organization (United Nations, 2015) on more than 7.5 million hectares that collectively produced up to 76 million tons of grapes in 2016 (OIV, 2016). Not only is grapevine cultivated under a range of vastly different climatic conditions, each vineyard is a notoriously variable environment in itself. Frequently, one vineyard consisting of one *V. vinifera* genotype may be representative of several smaller meso-climates depending on slope, soil composition as well as wind and sunlight exposure (Keller, 2010; Matese et al., 2014; Oyarzun et al., 2007). Furthermore, the variability of the microclimate of the grape bunches are highly diverse depending on the level of exposure to the surrounding meso-climate and canopy shading (Pereira et al.,

2006; Reshef et al., 2017). To further complicate the study of grapevine metabolism in a field setting, several viticultural practices are often implemented that alter the quality and quantity of light exposure to the vines and grapes (reviewed in Reynolds, 2010). Additionally, the genotype (cultivar) of the specific vines may play a distinct role in the mechanisms implemented to acclimate to fluctuating light (reviewed in Dai et al., 2011; Dal Santo et al., 2013).

The fact that *V. vinifera* is successfully cultivated despite this wide range of environmental conditions point towards the phenotypic plasticity of this species (Castagna et al., 2017). This plasticity refers to alteration of a plant's phenotype in order to ensure survival and fitness in a changing environment. The final composition of mature grapes at harvest is therefore a function of their genotype (G), the interaction with their immediate environment (E) and the vineyard management practices (M) implemented across the several months of development (GxExM). This phenotypic plasticity has recently gained considerable attention in the field of grapevine research (Anesi et al., 2015; Blanco-Ulate et al., 2015; Fortes and Gallusci, 2017; Joubert et al., 2016; Reshef et al., 2017; Dal Santo et al., 2013, 2016) since the grapevine genome sequence became available (Jaillon et al., 2007; Velasco et al., 2007).

This review will highlight the changes in the approaches followed towards studying the complexity of grapevine in a field setting and then summarize the current knowledge of how grapevine responds to variable light conditions. Furthermore, special attention will be given to the transcriptional and metabolic changes occurring in grape berries developing under varying light conditions and how viticultural manipulations may influence the outcome.

2.2 Multi-omics approaches towards integrating our knowledge on grapevine metabolism in the vineyard setting

It has been estimated that the cultivation of grapevine (*V. vinifera*) dates back to the approximately 6000 B.C. on the eastern shores of the Black Sea (Mullins et al., 1992). Ever since, viticultural practices have been implemented and optimized for the purpose of altering the outcome of the grapes by the final harvest date with highly variable outcomes (Imazio et al., 2013). This high level of variability resulted in the fact that grape cultivation was traditionally shrouded in unpredictability that is frequently conveniently attributed to seasonal effects. This "vintage effect" is frequently used in field-based grapevine studies and is further testament to the complexities of grapevine metabolism under highly variable growing conditions whereas "terroir" is the term used to collectively describe the outcome of the interaction between the vines, their environment and the effect of human intervention in a specific geographic location (reviewed in Fabres et al., 2017). Although a recent study has focused on deciphering the cryptic nature of the elusive "terroir" (Anesi et al., 2015), grouping the effect of a myriad potentially interacting variables together have provided an oversimplified impression of grape responses to the environment.

Similarly, viticultural manipulation, along with seasonal, diurnal and spatial fluctuations in light exposure to grapevines has raised many questions regarding how variable light effects grapevine metabolism. Traditionally, the effect of these viticultural practices were anticipated based upon anecdotally generated information or determined by comparing externally measured field conditions with measurements associated with grape quality based on yields or sugar concentrations of the grapes (Crippen and Morrison, 1986; Sommer et al., 1974). With the advent of more advanced technologies for the purpose of determining metabolite concentrations, more definitive links could be drawn between elevated exposure to grapes and the metabolic outcome of the treatment (Bertamini and Nedunchezhian, 2002; Dokoozlian and Kliewer, 1996; Haselgrove et al., 1992; Hashizume and Samuta, 1999; Jeong et al., 2004; Lee, 2017; Pereira et al., 2006; Riedel et al., 2015).

Since construction of the first consensus genome sequence for *V. vinifera* (Jaillon et al., 2007; Velasco et al., 2007), the integration of an additional layer of information on the gene level became possible. The genes involved in the synthesis of some of the most prominently light sensitive metabolites could be analyzed, leading to the extensive characterization of numerous targeted metabolic pathways, that were previously unexplored. Some of these pathways included in the study of grapevine responding to an altered microclimate involved the flavonoid compounds (Fujita et al., 2006; Guan et al., 2016; Jeong et al., 2004; Koyama et al., 2012; Liu et al., 2015; Matus et al., 2009; Zheng et al., 2013), isoprenoids (Sasaki et al., 2016; Zhang et al., 2014) and methoxypyrazines (Dunlevy et al., 2013; Gregan and Jordan, 2016), among many others (Bonomelli et al., 2004; Kühn et al., 2009; Zhang et al., 2014).

It was however, the advent of high-throughput gene expression technologies such as Microarray and RNA Sequencing (RNASeq) analyses in model plant systems that dramatically advanced our understanding of how plant metabolism may be influenced by light exposure on the gene level. The first ever microarray for the purpose of whole-transcriptome analysis in *Arabidopsis* was achieved by Schena et al., (1995). Soon after whole-transcriptome experimental technologies became available to study plant metabolism, the study of model plant systems revealed the high level of gene expression activity underlying some of the subtle metabolic changes associated with specific experimental treatments.

Examples in which whole-transcriptome analyses revealed underlying regulatory mechanisms include a publication by Jung et al., (2013), in which *Arabidopsis* leaves were exposed to elevated light. The authors targeted the expression of a gene encoding an ascorbate peroxidase (*APX2*) enzyme known to be involved in maintaining oxidative homeostasis under adverse abiotic conditions. By performing a whole-transcriptome microarray analysis, the authors identified a subset of heat shock protein (Hsp) and heat shock factor (Hsf) encoding genes that were upregulated up to a thousand fold in response to elevated light. The Hsfs were further implicated in the regulation of the *APX2* gene in question. The involvement of these Hsfs in plant response to elevated light may have gone unnoticed if targeted transcript analysis was performed instead.

In grapevine, the importance of multi-omics integration strategies have been recently reviewed in the context of how grapes respond to salinity stress (Daldoul et al., 2014) and as these technologies and their integration methodologies evolved, so did investigations into the effect of light on grapevine. Figure 2.2 represents the number of grapevine studies related to light manipulation published in the 21st century, making a distinction between the amount of studies that were performed in either a field setting or under controlled greenhouse/laboratory conditions. This figure shows that most of the studies that attempted to integrate several layers of data (generated from the same experimental grapevine tissues) were either conducted under controlled conditions in a greenhouse, or under notoriously variable conditions in the field. The details of the studies that reported data generated from both metabolic and transcriptomic analyses (targeted or whole-transcriptome) are further summarized in Table 2.1.

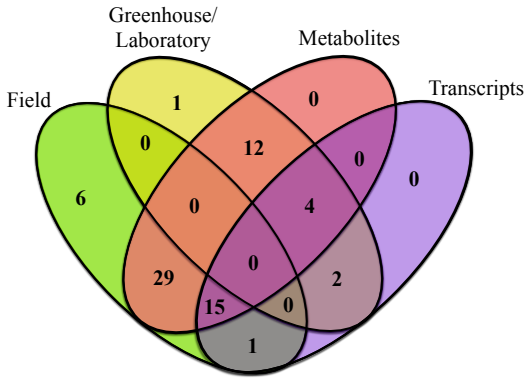

Figure 2.2. Venn diagram comparing the number of publications in the 21st century focused on the effect of light exposure on grapevine that generated either metabolic and/or transcriptomic data either in the field or under greenhouse/laboratory conditions.

Under greenhouse or laboratory conditions, researchers can control most of the growth parameters of the grapevine. Furthermore, this strategy allows for the complete characterization of the light treatment being implemented without taking other environmental and/or disease pressures into account. Three of the studies included in Figure 2.2 utilized these controlled conditions to generate results regarding the effect of either UV-A, UV-B (Martinez-Luscher et al., 2014; Zhang et al., 2013) or UV-C exposure (Bonomelli et al., 2004; Zhang et al., 2013). These studies revealed that grapes treated with UV-A, UV-B and UV-C exposure upregulated specific genes of the phenylpropanoid pathway that either elevated the accumulation of flavonols (Martinez-Luscher et al., 2014), flavan-3-ols (Zhang et al., 2013) and/or anthocyanins (Zhang et al., 2013). Zhang et al. (2013) further established that UV exposure differentially affects the gene expression and accumulation of flavan-3-ols depending on the developmental stage at treatment. Martinez-Luscher et al. (2014) showed that UV-B exposure does not induce higher anthocyanin accumulation in conjunction with water deficit stress despite higher accumulation of both of these metabolites under either UV-B or water deficit treatment separately. The authors concluded, similar to what has been reviewed recently (Pandey et al., 2015), that multiple stress exposures cause substrate competition between the enzymes responsible for abiotic stress responses. Furthermore, Bonomelli et al. (2004) showed that UV-C exposure

induces the expression of genes responsible for pathogenesis-related genes in grapevine leaves, hereby potentially enhancing resistance to fungal infection. Collectively, these greenhouse-based studies provided invaluable knowledge regarding how UV exposure may interact with other stress exposures in grapevine tissues by isolating specific factors.

The fourth study explored the entire transcriptome and amino acid profiles associated with diurnal oscillations (Rienth et al., 2014). This study was the first of its kind to report on the molecular patterns that form during the development of grape berries during night time and how these diurnal cycles influence berry development that would otherwise be problematic to study in isolation in a field setting. By minimizing the effects of confounding variables, the authors demonstrated that as much as 20% of the developmentally regulated genes were only expressed at night and that photoperiod regulation in grape berries undergo significant changes when the grapes transition towards ripening (Rienth et al., 2014).

It is however widely accepted that these highly controlled conditions can mask inherent crop characteristics and the clear differences in results generated from greenhouse and field grown plants have been reported in several plant systems. These plant species, among many others, include bell peppers (*Capsicum annuum*), in which treatment with growth promoting bacteria for the purpose of enhancing salinity stress tolerance was highly effective in greenhouses, but not in field applications (Bacilio et al., 2017). Another example includes three grass species (Heinze et al., 2016), in which the interactions of the plants and soil microorganisms were distinctly different under field and greenhouse conditions.

When studying the effect of light in particular, several challenges arise when comparing greenhouse and field-grown crops. Not only is there a profound difference in the quality and quantity of natural vs. artificial lights but natural light is constantly fluctuating based on the time of day (diurnal and seasonal patterns), as well as potential cloud cover. These differences may be the cause for differences reported in the morphology and pigment accumulation when comparing *Arabidopsis* leaves grown in a greenhouse with those grown outdoors (Küllheim et al., 2002; Mishra et al., 2012).

Table 2.1. A summary of the 21st century publications focused on the effect of light exposure in grapevine that integrated results generated from metabolite and transcript measurements.

Type of studies	Authors	Genotype (cultivar)	Tissue	Metabolite measurements	Transcript analysis	Treatment
Greenhouse/ Laboratory studies	Bonomelli et al., 2004	Chardonnay	Detached Leaves	Resveratrol, Chitinase, β-1,3-glucanase	Rt-PCR	UV-C treatment
	Zhang et al., 2013	Cabernet sauvignon	Detached Berries	Flavan-3-ols	Rt-PCR	UV-A, UV-B treatment
	Reinth et al., 2014	Microvine	Berries	Amino acids	Microarray, Rt-PCR	Diurnal sampling patterns
	Martínez-Lüscher et al., 2014	Tempranillo	Berries	Phenylpropanoids/ Flavonoids	Rt-PCR	UV-B treatment
Field studies	Jeong et al., 2004	Cabernet sauvignon	Berries	Anthocyanins	Rt-PCR	18-20% shading of berries
	Matus et al., 2009	Cabernet sauvignon	Berries	Anthocyanins	Rt-PCR	Leaf removal in the bunch zone
	Koyama et al., 2012	Sauvignon blanc	Berries	Phenylpropanoids/ Flavonoids	Rt-PCR	Sunlight exclusion of berries
	Zheng et al., 2013	Jingxiu, Jingyan	Berries	Anthocyanins	Rt-PCR	Sunlight exclusion of berries
	Pastore et al., 2013	Sangiovese	Berries	Phenylpropanoids/ Flavonoids	Microarray, Rt-PCR	Leaf removal in the bunch zone
	Dunlevy et al., 2013	Cabernet sauvignon	Berries	Methoxypyrazines	Rt-PCR	Sunlight exclusion of berries
	Fujita et al., 2014	Cabernet sauvignon, Merlot	Berries	Flavonols	Rt-PCR	Shading of the bunches
	Wu et al., 2014	Jingxiu, Jingyan	Berries	Anthocyanins	RNASeq, Rt-PCR	Sunlight exclusion of berries
	Carbonell-Bejerano et al., 2014	Tempranillo	Berries	Phenylpropanoids/ Flavonoids	Microarray	Leaf removal in the bunch zone; UV transmitting/excluding screens in the bunch zone
	Liu et al., 2015	Sauvignon blanc	Berries	Flavonols	Rt-PCR	UV transmitting/excluding screens in the bunch zone
	Guan et al., 2016	Gamay, Gamay Fréaux	Berries	Anthocyanins	Rt-PCR	Sunlight exclusion of berries
	Sasaki et al., 2016	Sauvignon blanc	Berries	Terpenes	Rt-PCR	Sunlight exclusion of berries
	Gregan and Jordan, 2016	Sauvignon blanc	Berries	Methoxypyrazines	Rt-PCR	Leaf removal in the bunch zone
	Sun et al., 2017	Cabernet sauvignon	Berries	Phenylpropanoids/ Flavonoids	RNASeq, Rt-PCR	Leaf removal in the bunch zone
	Zenoni et al., 2017	Sangiovese, Ortrugo, Ciliegiolo, Nero d'Avola	Berries	Phenylpropanoids/ Flavonoids	Microarray, Rt-PCR	Leaf removal in the bunch zone

Additionally, growing grapevines to the level of maturity required to study their reproductive tissues and fruit in a greenhouse setting may be problematic due to space, time and other constraints. To this end, plant tissues are frequently detached in the field for experimental purposes in the laboratory. Examples of this approach are depicted in Table 2.1, in which the authors made use of detached leaves (Bonomelli et al., 2004) and grape berries (Zhang et al., 2013) to study the effects of UV. This experimental strategy may however, potentially initiate systemic wound responses in the detached tissues that may mask the outcome of the results. This approach may further have limited success when studying the long-term effects of light exposure since detached tissues cannot be assumed to retain metabolic functioning representative of their functioning as part of the whole-plant metabolism long after detachment.

Alternatively, the use of fruit bearing canes is frequently implemented as a model for the study of grape tissues under controlled environmental conditions as initially described by Mullins et al., (1966). According to this model, the development of grapevine tissues can be artificially coordinated from dormant cuttings for the purpose of performing experiments under controlled conditions. Although several studies have reported the success of this experimental model in the investigation of berry development and the effect of specific treatments (Dai et al., 2013; Martínez-Lüscher et al., 2015a, 2015b), some discrepancies between berries grown from cuttings and those from field-grown vines have been reported. The results from Dai et al. (2013) revealed that although the sugars and organic acid contents of grapes developed under these two conditions

were highly similar, their results also proposed that the grapes were distinctly different in the flux between the pathways associated with central carbon metabolism due to significant differences in the accumulation of phosphorylated precursors. The authors acknowledged that these differences between field-grown vines and fruit bearing cuttings may reflect how the field grown grapes respond uniquely to short-term fluctuations in environmental exposures, such as light (Dai et al., 2013). Therefore, although greenhouse studies may provide an invaluable foundation of knowledge regarding specific metabolic processes in plants, these earlier reports show that the transfer of knowledge from greenhouse studies towards field applications may prove to be problematic.

Currently, one of the more effective models to facilitate the study of whole-plant and grape berry development in a controlled setting is the use of the Microvine system (Chaïb et al., 2010). The microvine materials originated after the description of a *V. vinifera* mutant that has a dwarf stature, continuous flowering and relatively short generation cycles due to a mutation in the *GA1* gene (Boss and Thomas, 2002). The features of the microvines provide an accelerated mechanism to study grapevines under controlled conditions. Most recently, the effectivity of utilizing microvine in the study of grapevine leaves, flowers and berries responding to modulated temperatures were evaluated (Luchaire et al., 2017). The authors reported that microvine showed similar vegetative and reproductive developmental patterns when compared to other grapevine cultivars and concluded that it provides an excellent model for studying abiotic stresses. Previous publications with the microvine that focused on studying the diurnal patterns (Rienth et al., 2014) and the effect of temperature in grapevine (Rienth et al., 2016) further showed the effective implementation of this model system.

In studying grape berries, the approach that has been more frequently taken involves the evaluation of berries developing under various conditions in the field (Table 2.1). Although this approach more accurately depicts the true responses of grapes to their environment, field-studies prove to be notoriously challenging. As previously mentioned, vineyards are highly variable and developing grapes are challenged by a myriad of confounding environmental variables, exacerbated by the viticultural manipulations implemented during their development, hereby making the quantification of grape responses to one specific condition, such as light, extremely difficult.

One of the most commonly applied viticultural practices is the removal of leaves in the vine canopy at various stages throughout the development of the grapes, hereby altering the berry microclimate. This application is associated with several advantages. These include aiding in pest management by lowering canopy humidity and therefore fungal infection (Chellemi and Marois, 1992; English et al., 1989), providing a balance in crop load (Palliotti et al., 2012) and making the fruit more accessible during harvest. Additionally, the quality and sensory attributes of grapes grown on defoliated vines are known to be different from shaded grapes. These altered quality parameters include balanced pH levels in grape juices (Hunter and Visser, 1990; De Toda et al., 2013) and a reduction of green aroma characteristics in the

resulting wines (Šuklje et al., 2014). Furthermore, in red wine cultivars, an improvement in color stability is also reported in grapes exposed by leaf removal (Chorti et al., 2010; Lee and Skinkis, 2013). However, this common viticultural practice of leaf removal that allows for elevated light exposure to the grapes very rarely effect levels of light without elevating bunch temperatures as well. To this end, researchers predominantly follow one of two approaches when studying the effects of leaf removal in the berry bunch zones.

The first approach involves making no attempt at separating the effects of temperature and light, but to rather follow the transcriptional and/or metabolic changes that occur in response to the treatment itself (exposure). Examples of this approach are published in Pastore et al., (2013) and Zenoni et al., (2017) in which the authors performed a whole-transcriptome and targeted metabolite analysis comparing shaded grapes to grapes exposed by leaf removal treatments. The authors reported on the effect that leaf removal at different berry developmental stages had on berry skin temperatures and although they showed that exposed grapes had higher levels of external sunburn damage (Pastore et al., 2013), they did not report on the levels of light the grapes were exposed to in response to the treatment.

The second approach frequently followed in the study of field-grown grapes is attempting to focus on the effect of light exposure on the grapes without taking temperature into account. An example of this approach was taken by Sun et al., (2017) in which the authors linked the effect of sunlight exposure on the whole-transcriptome and accumulation of phenolic compounds in developing Cabernet Sauvignon grapes. In this study, the authors reported and acknowledged the effect that the leaf removal treatment had on the berry cluster temperatures during three consecutive years (Supplementary Table 1; Sun et al., 2017), however, clear links between how the temperature may have affected the differential accumulation of flavonoid compounds were not drawn. The authors further reported a vintage effect regarding the accumulation of these compounds. Other examples of this effect is reported in Downey et al., (2004) in which the authors reported how the accumulation of anthocyanins were significantly affected by light exclusion one year but not during the other two vintages studied in their investigation.

Although both of these approaches contribute to our current understanding of how grape berries respond to leaf removal in general, it does not provide conclusive information about how grapes respond to light in particular. It has further been shown that the metabolic pathway most frequently studied in the context of light exposure, i.e. the flavonoid biosynthesis pathway, is differentially affected by light and temperature conditions in detached grapes (Azuma et al., 2012). Therefore, in order to successfully link light exposure to a specific physiological, metabolic and transcriptional response in the grapes, it would be necessary to characterize other possible confounding variables, such as temperature, as well.

In order to address this challenge, the term “field-omics” was put forward to describe an experimental approach that could potentially eliminate the effect that multiple confounding variables may have on the outcome of field-based research (Alexandersson et al., 2014). According to this approach, several omics

disciplines should be combined to best characterize the genomic, transcriptomic and metabolomic outcomes of a specific field condition in order to bridge the current gap between crop genotype and the effect of its environment on the ultimate phenotype. This experimental design strategy further promotes the idea that the characterization of the plot layout and effective sampling strategy will contribute significantly to effectively limit the effects that vineyard variability may have on the outcome of field-based study results. The relevance and success of this multi-data integration approach is highlighted by recent studies that have further contributed to the improvement of grapevine field experimental systems (Carbonell-Bejerano et al., 2016; Reshef et al., 2017). One of these studies commenced the tedious task of unraveling the inherent heterogeneity within grape berry clusters (Reshef et al., 2017). This study revealed that by integrating micrometeorology and metabolomics, the effect of sunlight on the development of specific metabolites accumulate uniquely based on the location within the berry bunch (Reshef et al., 2017). Another study focused on developing a berry density sorting method that could potentially reduce the heterogeneity of grapes samples by synchronizing the developmental stage of the grapes (Carbonell-Bejerano et al., 2016).

These pioneering multi-omics studies acknowledged the necessity for such highly integrated experimental strategies and the implementation of such strategies are becoming more prevalent in grapevine field research. Further examples of such highly characterized conditions for the purpose of studying light exposure include Koyama et al., (2012) and Guan et al., (2016) represented in Table 2.1. Both of these studies utilized opaque boxes to exclude almost all sunlight from the microclimate of the grapes. Furthermore, measurements taken in the berry microclimates concluded that these treatments did not affect temperature, hereby excluding temperature as a possible confounding variable (Guan et al., 2017; Koyama et al., 2012). Therefore, these studies provided clear insights into how various light conditions specifically affect the genes and metabolites of the phenylpropanoid/flavonoid pathways in developing grapes.

Two of the most recent studies performed on field-grown grapes (represented in Table 2.1) combined transcript and metabolite data and utilized whole-transcriptome analysis techniques (Sun et al., 2017; Zenoni et al., 2017). Utilizing five different omic technologies in parallel, Ghan et al., (2015) aimed to establish the level of success of each of the whole-transcriptome analysis platforms when comparing NimbleGen Grape Whole-Genome Microarray to the Illumina RNASeq technologies in five grapevine cultivars simultaneously. Through this exhaustive experimental procedure, the authors established that a larger number of proteins were significantly correlated with the RNASeq data than with the Microarray data (Ghan et al., 2015), however, the RNASeq technology is not devoid of shortcomings. In grapevine research, the sequence reads generated from RNASeq is mostly aligned to the Pinot noir reference genome (PN40024) regardless of which cultivar is being studied. It was subsequently established that disruptive mutations could have affected up to one third of the proteins coded for by the PN40024 genome when evaluated in the *V. vinifera* cultivar, Corvina (Venturini et al., 2013). The authors further identified as many as 180 genes unique to the Corvina cultivar through the *de novo* assembly of the Corvina transcriptome. Through these findings, the authors pointed towards the unique transcriptional signatures that underlie the high level of

phenotypic divergence among grapevine cultivars, despite the low level of genomic heterogeneity among grapevine cultivars (Myles et al., 2011). In the future, the *de novo* assembly of specific genotypes should not simply be reserved for species that lack the availability to a reference genome but should also be considered for species that have a complex domestication history, such as *V. vinifera*. Furthermore, the development of a novel sequencing platform, PacBio SMRT, aims to revolutionize the manner in which the genomes of diploid or polyploidy plant species, such as *V. vinifera*, are studied (Chin et al., 2016).

Additionally, the concept of constructing pan-genomes may further account for the sequencing problems associated with highly divergent and rearranged genomes by generating a more complete sequence from multiple sequenced genomes of various genotypes within one species (for review, see Wendel et al., 2016). This concept has proven to be highly informative in the evaluation of the maize pan-genome and pan-transcriptome (Hirsch et al., 2014) and may provide an invaluable resource in the future of grapevine molecular biology. Accordingly, the transcriptional signatures associated with specific grapevine varietal characteristics in response to their specific biotic and abiotic environments are expected to be understood with greater accuracy in the future. In the remainder of the review, the impact of light on grapevine metabolism will be summarized, according to current literature.

2.3 Grapevine metabolism and the impact of light

In grapevine, the effect of elevated light and particularly UV irradiation has been studied in both foliar (vegetative) tissues as well as in grape berries. Early studies focusing on the effect of light exposure identified the synthesis of high levels of the stilbene, resveratrol, when grapevine leaves were exposed to UV light (Langcake and Pryce, 1976). These earlier findings were instrumental in the identification and characterization of several resveratrol analogs that are now known to respond to UV exposure (Pezet et al., 2003). Kolb et al. (2001) further explored the metabolic responses of grapevine leaves to UV-B and identified the inhibitory effect that excessive exposure has on the efficacy of leaf photosynthesis. These findings were later supported by a study that confirmed the inhibitory effect of short term UV-B exposure on grapevine leaves (Martínez-Lüscher et al., 2013). This study further reported that long term UV-B exposure instead leads to an acclimated state through alterations to the leaf pigment and antioxidant composition.

Findings from transcriptome studies further elaborated on how foliar grapevine tissues respond to variations in light exposure. Taking a targeted approach, Bonomelli et al. (2004) showed that grapevine leaves respond to UV-C treatment by activating genes involved in defense responses that were further mirrored by the accumulation of stilbenes and glucanases. Additionally, several global transcriptome studies revealed which metabolic pathways were most significantly affected by either high or low UV-B treatments (Pontin et al., 2010), high light exposure (Carvalho et al., 2011), UV-C exposure of grapevine leaves (Xi et al., 2014) or the effect that blue or compound light emitting diode lights may have on whole grapevine plantlets grown *in vitro* (Li et al., 2017). Although each of these metabolic and/or transcriptomic studies contributed unique

insights into how grapevine leaves respond to variations in light exposure, these studies collectively revealed similar results and indicated that the metabolic processes involved in the phenylpropanoid/flavonoid synthesis pathways and the down-stream metabolites of these pathways, such as hydroxycinnamic acids, flavonols and stilbenes, are highly sensitive to levels of and/or type of light exposure in grapevine leaves.

Green fruit, such as green grapes, are proposed to behave much like other foliar tissues such as leaves (reviewed in Blanke and Lenz, 1989). However, it was the grapevine gene expression atlas that generated some of the first profiling analyses and showed that green berries behave transcriptionally more similar to other green, vegetative tissues such as leaves, tendrils and buds, compared to ripening berries (véraison onwards) (Fasoli et al., 2012). Interestingly, the effect that light has on the ripening stages of berry development has enjoyed more attention in recent studies (Carbonell-Bejerano et al., 2014; Guan et al., 2016; Jeong et al., 2004; Matus et al., 2009; Wu et al., 2014; Zheng et al., 2013) compared to efforts to understand how green grapes and foliar tissues might overlap in their transcriptional and metabolic responses to increases in light exposure; the latter leaving a gap in our understanding that warrants further attention.

Our knowledge of how grape berries respond to light in their immediate microclimate hinges on our current understanding of the intricate details regarding their development and ripening program. Several studies have contributed to our current understanding of this complex developmental process through detailed investigations into the physical and biochemical changes that takes place throughout berry development (Conde et al., 2007; Coombe and McCarthy, 2000). These changes were visually represented by Coombe and McCarthy, (2000) and recently reviewed in Fortes et al., (2015). The formerly mentioned visual representation is depicted in Figure 2.3 (Serrano et al., 2017).

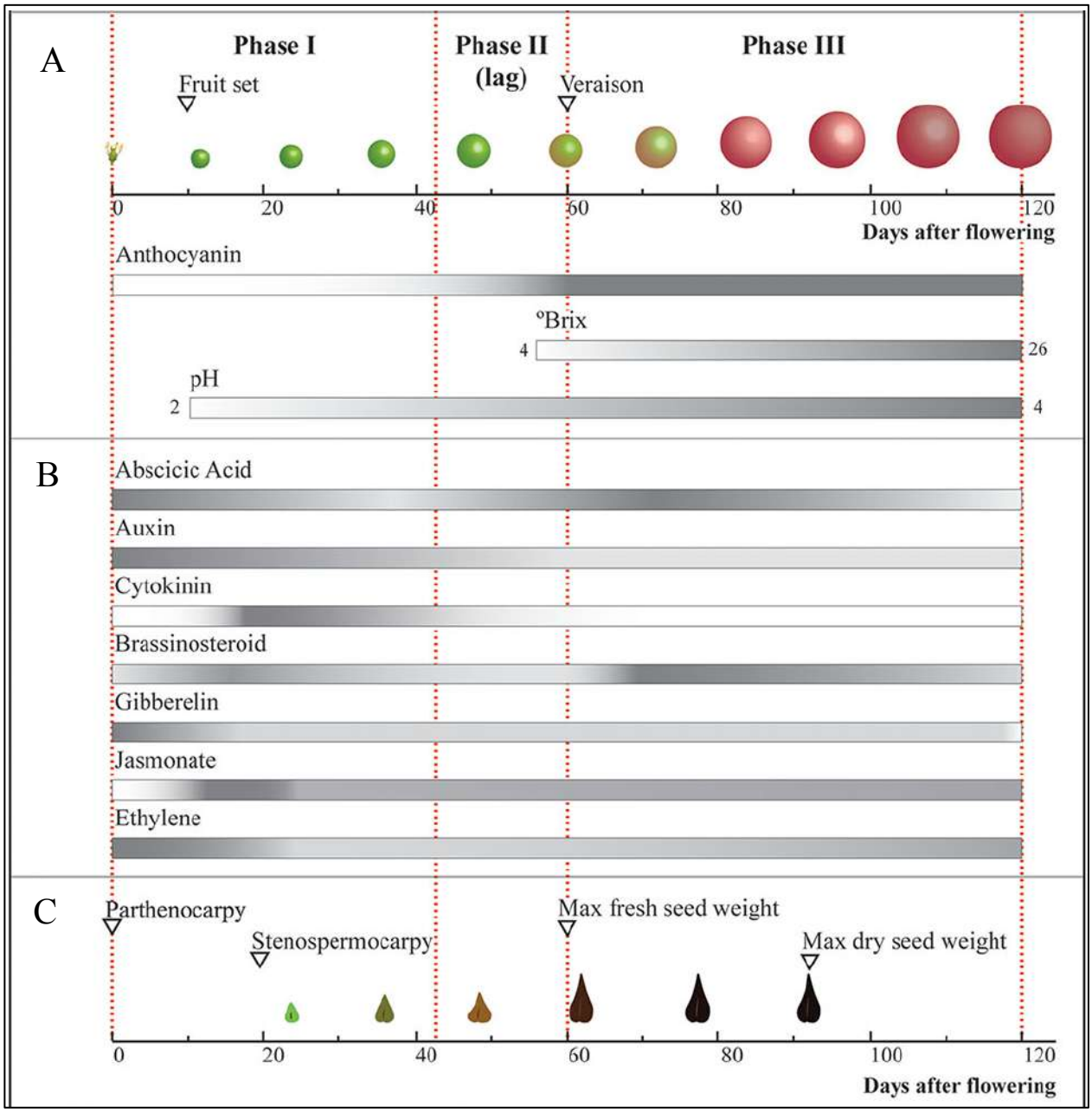

Figure 2.3. Grape berry development (Serrano et al., 2017) represented as a diagram depicting the most important changes that grape berries and seeds undergo during development. (A) Changes in size, color, Brix degree and pH during berry development. (B) Variations in hormonal content during grape berry development. (C) Seed development showing the time in which parthenocarpy and stenospermocarpy can take place. The main events are indicated by open triangles. Bars represent the changes throughout development, in which gray and white represent the higher and lower estimated referential values for each parameter, respectively.

Due to the economic importance of grape berries as a fruit crop, the necessity to fully characterize this highly complex developmental program encouraged numerous studies that focused on its characterization on a molecular level. Some of the first studies focused on characterizing the gene expression patterns associated with grape berry development. These studies initially targeted expression levels of specific genes putatively associated with invertase activity involved in sugar accumulation (Davies and Robinson, 1996), anthocyanin biosynthesis (Boss et al., 1993), chitinases (Robinson et al., 1997) and how some of these developmentally

regulated genes are affected by phytohormone (auxin) treatment (Davies et al., 1997). These studies contributed to the elucidation of the underlying molecular mechanisms associated with several metabolic pathways regulated by berry development (reviewed in Robinson et al., 2000) although more recently Pilati et al., (2017) identified abscisic acid (ABA) to be the major regulator in the berry developmental program. The authors identified a 71% overlap between developmentally regulated genes and genes upregulated in response to ABA treatment and that even a small amount of ABA exposure could trigger higher ABA accumulation and the modulation of as many as 1893 genes (Pilati et al., 2017).

Directly after the grapevine genome sequence became available (Velasco et al., 2007; Jaillon et al., 2007), two studies using microarray technologies reporting on the global gene expression patterns associated with the development of grape berries were published (Deluc et al., 2007; Pilati et al., 2007). One of these studies provided the first high-resolution overview of the underlying transcriptional activity during seven stages measured throughout the development of Cabernet Sauvignon grapes (Deluc et al., 2007). Similarly, the other publication reported on the global transcriptional activity over the developmental time-course of Pinot Noir grapes and further established the occurrence of an oxidative burst that takes place at the onset of ripening (Pilati et al., 2007). With the advent of RNASeq technology, the study of molecular mechanisms associated with the development of various grape cultivars were further refined (Sweetman et al., 2012; Zenoni et al., 2010).

Subsequently, several studies aimed to characterize the transcriptional switches that occur when the berries are transitioning from one developmental stage (for example green) to another (for example ripening) (Fasoli et al., 2012; Palumbo et al., 2014; Zamboni et al., 2010). Zamboni et al., (2010) developed an analysis pipeline that allowed them to identify developmental stage-specific molecular biomarkers by integrating several omics datasets generated from the same grape berry samples throughout development. Based on this approach, the authors successfully provided not only an analysis pipeline for future work, but identified several molecular biomarkers with which to compare future multi-omics studies involving developing grapes. These findings supported earlier targeted gene expression studies regarding the transcription of cell-wall associated genes during the early developmental stages (Davies and Robinson, 2000) and the role of phenylpropanoid pathway gene expression during the ripening stages (Boss et al., 1993). Among the numerous novel outcomes of this pioneering study was the discovery that sphingolipid fatty acids potentially act as signaling molecules during the early developmental stages.

Utilizing the wealth of data generated in the grapevine gene expression atlas (Fasoli et al., 2012), Palumbo et al. (2014) further identified and adopted the term “switch genes”. These genes, that include some specific transcription factor encoding genes, were proven to behave as master regulators in the metabolic shift that takes place between green and ripening stages in grapes. These conclusions were driven by the fact that they show somewhat opposite expression patterns when comparing green to ripening berry transcriptomes. Aside from the transcription factors implicated in the regulation of this transition, several of these switch genes are

involved in carbohydrate metabolism and secondary metabolism involved in both the phenylpropanoid pathway and the degradation of carotenoids (Palumbo et al., 2014).

These studies therefore provide a foundation with which to compare gene expression at specific grape developmental stages to determine whether the development of the berries under investigation is progressing according to the established profile. Furthermore, the expression of a predetermined set of genes that characterize each grape developmental stage could hold valuable advantages in future investigation into grape berry metabolism. By identifying these predetermined developmentally driven gene expression profiles, researchers might be able to more accurately distinguish developmental patterns from those induced by a specific treatment or stress conditions.

2.3.1 Grape berry metabolism responds strongly to light modulation

Green fruits, including green grape berries, are photosynthetically active (reviewed in Blanke and Lenz, 1989) and the effect that light fluctuations may have on the primary metabolism of grapes have been comprehensively reported: elevated exposure typically lead to increased accumulation of sugars, and decreased acidity in grapes (reviewed in Reynolds, 2010). Sunlight exclusion on the other hand is known to lower berry weight and total soluble solids of the berries by harvest, while titratable acidity was elevated (Dokoozlian and Kliewer, 1996; Zhang et al., 2014, 2017).

Similarly, some well-established concepts with regards to light quantity (exposure/exclusion), or quality on some secondary metabolites (such as anthocyanins, flavonoid compounds and carotenoids/apocarotenoids) have been established for grapevine berry development and ripening, but it is clear that the impacts of light on the broader metabolism, and specifically the integration of primary and secondary metabolism is not fully understood.

Secondary metabolites are synthesized through the energy and precursors provided by central carbon metabolism as part of primary metabolism. These secondary metabolites are not only involved in the normal development of grape berries but are frequently synthesized in response to various biotic and abiotic stimuli in a development-independent manner. Interestingly, many of the secondary compounds that have been shown to be modulated by abiotic and biotic stresses are linked to perceived quality-related impact factors for grape and wine quality. Wine quality depends on the complex balance between primary and secondary metabolites and the countless combinations in which they accumulate in the grape berries throughout development (Herderich et al., 2012; Jackson and Lombard, 1993). The link between primary metabolism, the precursors involved and these secondary metabolic pathways are depicted in Figure 2.4. Due to their close association with the sunlight-dependent primary metabolic process of photosynthesis and the importance of these compounds in berry composition, it is not surprising that the accumulation of several of these compounds are frequently studied in the context of variable light exposure to grape berries. Among the

secondary metabolic pathways closely associated with primary metabolism is the synthesis of isoprenoids, the downstream metabolism of amino acids that lead to the synthesis of phenylpropanoids, branched chain aliphatics and methoxypyrazines and the degradation of fatty acids that provide precursors to volatile thiols, liberated during alcohol fermentation. These pathways and their links to light modulation in berries will be summarized in the next sections.

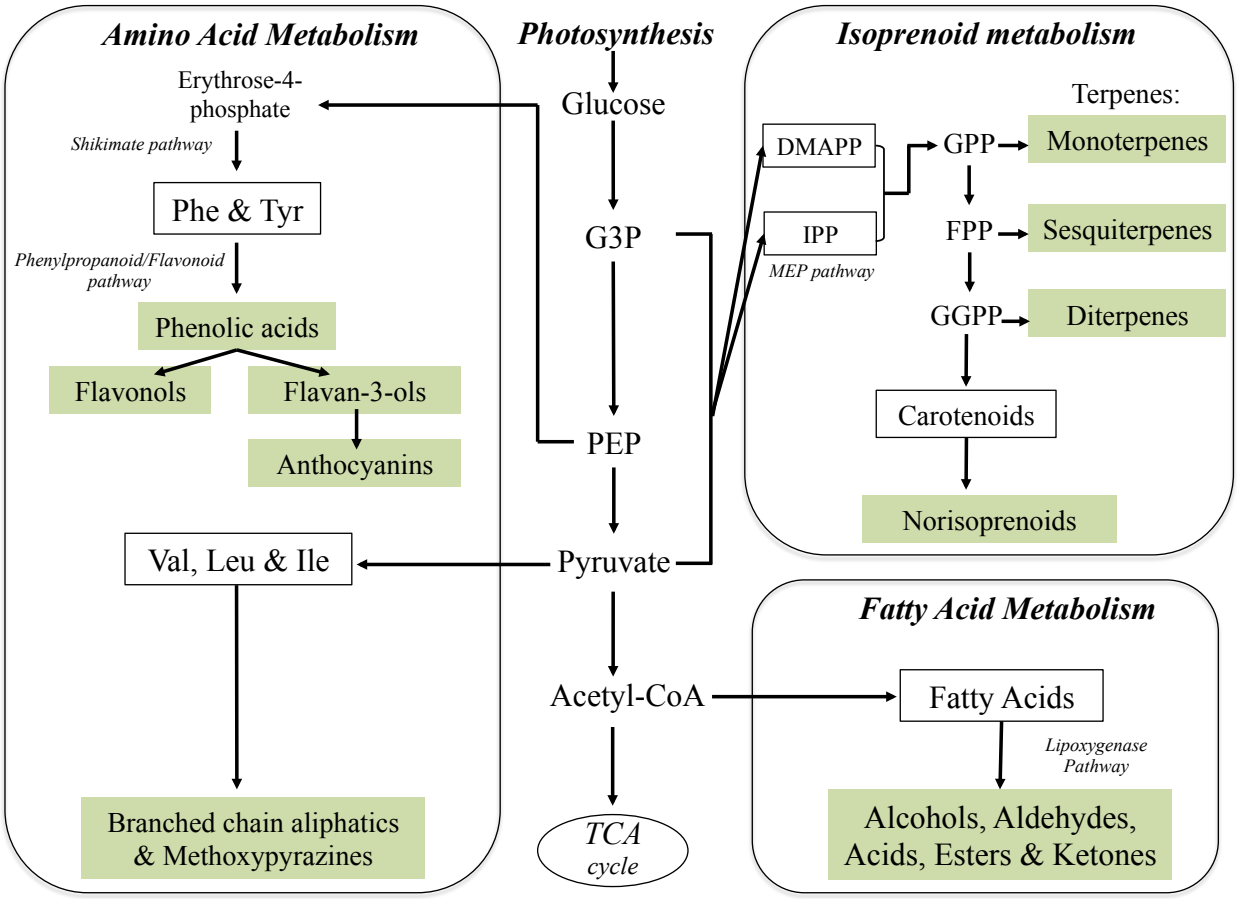

Figure 2.4. Biosynthetic pathways of three classes of volatile compounds in grapes (adapted from Liu et al., 2015). Metabolism and pathway genes names are italicized, volatiles are shaded in green. Abbreviations: MEP, 2-C-methylerythritol-4-phosphate; G3P, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvic acid; Acetyl-CoA, acetyl coenzyme-A; TCA, tricarboxylic acid; DMAPP, dimethylallyl pyrophosphate; IPP, isopnetenyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; Phe, phenylalanine; Tyr, tyrosine; Val, valine; Leu, leucine; Ile, Isoleucine.

2.3.1.1 Isoprenoid metabolism and berry light modulation

In the metabolic process of isoprenoid synthesis in plants, the two precursors, dimethylallyl pyrophosphate (DMAPP) and isopentyl pyrophosphate (IPP), can be formed from either the mevalonic acid (MVA) pathway in the cytosol (reviewed in Newman and Chappell, 1999) or via the 2-C-metylerythritol-4-phosphate (MEP) pathway in plastids (Rohmer et al., 1999). The isoprenoids synthesized from these precursors are terpenes and isoprenoids, respectively (2.4).

Terpenes are crucial contributors to primarily the floral and fruity notes of grape and wine aroma through their presence in either free or glycosylated form (Mateo and Jimenez, 2000). The evolution and accumulation of terpenes throughout the various stages of berry development appears to be cultivar specific. In two Muscat varieties (Muscat d'Alexandria and Muscat de Frontignan), the accumulation of terpenes increases from pre-véraison until the berries are ripe (Gunata et al., 1985; Park et al., 1991), similar to a more recent study on the table grapes, Jingxiangyu (Zhang et al., 2017). In contrast, Riesling and Cabernet Sauvignon grapes reached maximum levels of terpene accumulation pre-véraison, after which concentrations declined (Kalua and Boss, 2010; Zhang et al., 2017).

As early as the 1980s, altered terpene levels in grapes were reported in response to leaf removal (and presumably light exposure) in the vineyard (Reynolds and Wardle, 1988, 1993; Smith et al., 1988; Reynolds et al., 1996). Ever since, as experimental procedures were refined, it was reported that bunch shading and exposure to solar and/or UV light resulted in altered monoterpene profiles in the grapes (Belancic et al., 1997; Bureau et al., 2000; Friedel et al., 2016; Joubert et al., 2016; Zhang et al., 2014; Zhang et al., 2017). Additionally, the grape genes and enzymes involved in terpene synthesis, terpene synthases, were functionally characterized (Lücker et al., 2004; Martin and Bohlmann, 2004; Martin et al., 2010, 2012). Subsequent studies have aimed to characterize the effect of light exposure on targeted transcript and metabolite level, revealing that transcription and accumulation of linalool, a monoterpene, and its oxides (hotrienol, *trans*-furan linalool oxide and *trans*-pyran linalool oxide) is the most sensitive to light in grapes (Friedel et al., 2016; Zhang et al., 2017). Zhang et al., 2017 further showed that expression of upstream genes involved in the MEP pathway are also sensitive to light, similar to results produced in *Arabidopsis* (Carretero-Paulet et al., 2002).

As these reports revealed, terpenes are predominantly studied in the context of grape and wine aroma profiles. It is further well established that terpenes (especially monoterpenes) predominantly serve the physiological purpose of either deterring pathogens and herbivores or contributing to the aroma profiles of fruit in an effort to attract pollinators and seed dispersers (reviewed in Unsicker et al. 2009). However, the physiological role of these terpenes in grape berry protection against variable light exposure remains relatively unexplored, although their roles in oxidative stress homeostasis have been studied in other experimental systems. Oxidation assays revealed the ability of several terpenes to scavenge free radicals (Wei and Shibamoto, 2007) after the antioxidant activity of terpenes was proposed in *Arabidopsis* (Aharoni et al., 2003). It was subsequently established that isoprene has the ability to protect plant membranes from oxidative stress (Siwko et al., 2007). A more recent study confirmed an additional role of terpenes in the stabilization of plant membranes in the African species, *Xerophyta humulis*, as part of the plant's mechanisms to survive dehydration stress (Beckett et al., 2012). Future studies focused towards the potential roles of terpenes in grape berry responses to fluctuating light are needed to elucidate the molecular mechanisms involved.

As part of isoprenoid metabolism, norisoprenoids (apocarotenoids) are products of either photochemical and/or enzymatic degradation of carotenoids. In addition to the primary light harvesting pigments (chlorophylls), these carotenoids are accessory light harvesting pigments involved in photosynthesis and are synthesized from isopentenyl pyrophosphate (IPP; Figure 2.5). Carotenoid pigments are either carotenes, or their oxygenated derivatives known as xanthophylls and are responsible for the color and aromas of several plant tissues such as flowers and fruits, respectively.

These carotenoids and their synthesis and degradation pathways form an indispensable part of plant metabolism and are highly conserved among photosynthetic organisms including plants, algae and cyanobacteria (reviewed in Cunningham and Gantt, 1998). In grapevine, the enzymes and metabolites involved in carotenoid synthesis were characterized (Baumes et al., 2002; Razungles et al., 1996; Young et al., 2012) and it was established that that β-carotene and lutein are the predominant carotenoids in grapes and are known to accumulate at high levels before véraison, after which they decrease dramatically (Crupi et al., 2010; Joubert et al., 2016; Leng et al., 2017; Mendes-Pinto et al., 2005; Fernandes de Oliveira et al., 2003; Razungles et al., 1988; Razungles et al., 1996; Ristic et al., 2007; Young et al., 2012; Yuan and Qian, 2016). This decrease coincides with the photochemical and/or enzymatic degradation of these carotenoids to form apocarotenoids (norisoprenoids). These norisoprenoids fulfill several physiological functions in plant metabolism. They can act as phytohormones such as abscisic acid and strigolactone, they are known to contribute to the varietal impact odorants of grapes and wines (Razungles et al., 1996; Mendez-Pinto et al., 2009; Crupi et al., 2010; Young et al., 2012) and more recently, they have been established as stress signaling molecules with the bioactivity required to initiate the expression of stress-related genes involved in plant stress acclimation (for review, see Havaux et al., 2013). Therefore, carotenoids synthesized during the early stages of grape berry development serve as precursors for the accumulation of phytohormones and aroma precursors (norisoprenoids) in ripe grapes under normal growing conditions. These seminal studies paved the way for the characterization of the genes and enzymes of the carotenoid biosynthetic pathway (Figure 2.5; Young et al., 2012).

When light exposure exceeds levels necessary to sustain plant growth, carotenoids are implicated in photoprotection through their involvement in two separate mechanisms. Firstly, they are involved in the protection of the photosynthetic machinery through non-photochemical quenching (NPQ) in an attempt to avoid photodamage though the dissipation of excessive excitation energy. Through the process of NPQ, the xanthophyll cycle is activated in which the xanthophyll pigment, violaxanthin, is de-epoxidized to zeaxanthin through the activity of the violaxanthin-deepoxidase (VDE) enzyme, hereby limiting energy transfer from the light harvesting complex II to photosystem II (reviewed in Müller et al., 2001; Niyogi and Truong, 2013). This NPQ mechanism could be considered the plant's first line of defense against photodamage. Secondly, some carotenoids, such as β-carotenes, as well as apocarotenoids, are capable of quenching ROS themselves through antioxidant activity (Telfer et al., 1994). Although it was previously established that carotenoid metabolism is highly conserved among plants, these photoprotective mechanisms

have been predominantly characterized in foliar/vegetative plant tissues. Green fruit retains modified photosynthetic activity (Blanke and Lenz, 1989) and these photoprotective mechanisms in tomato have been extensively characterized (reviewed in Bramley et al., 2002). However, the tomato ripening dynamics differ significantly from that of grape berries in that chloroplast plastids are transformed into chromoplasts during tomato ripening (Piechulla et al., 1987) and the mechanisms involved are therefore not directly comparable.

In grapevine, not only are these carotenoids compounds of interest in the study of photoprotection and acclimation to environmental stress, but the mechanisms with which grapes utilize these compounds may hold organoleptic advantages in winemaking. Surprisingly, despite the tight-knit association between carotenoids and the efficient functioning of the photosynthetic machinery, research focused towards the effect that light exposure may have on carotenoid and isoprenoid accumulation throughout grape berry development remains fairly limited.

Some studies have been performed by comparing the norisoprenoid contents of grapes and wines that were either shaded or exposed by a leaf removal treatment. These studies did however, report conflicting results with some studies revealing higher levels of the norisoprenoids (Ristic et al., 2007; Bureau et al., 2000; Zoecklein et al., 2008) whereas several others reported the opposite result (Lee et al., 2007; Kwasniewski et al., 2010; Marais et al., 1992). A more recent leaf removal study conducted over a three year period analyzed the isoprenoid content of ripe Pinot noir grapes exposed by various degrees of leaf removal in the berry bunch zone (Feng et al., 2015). The authors concluded that elevated β-damascenone levels were in fact correlated with the degree of leaf removal implemented and therefore the amount of light exposure, hereby possibly explaining the conflicted reports from studies inevitably performed under different levels of light exposure.

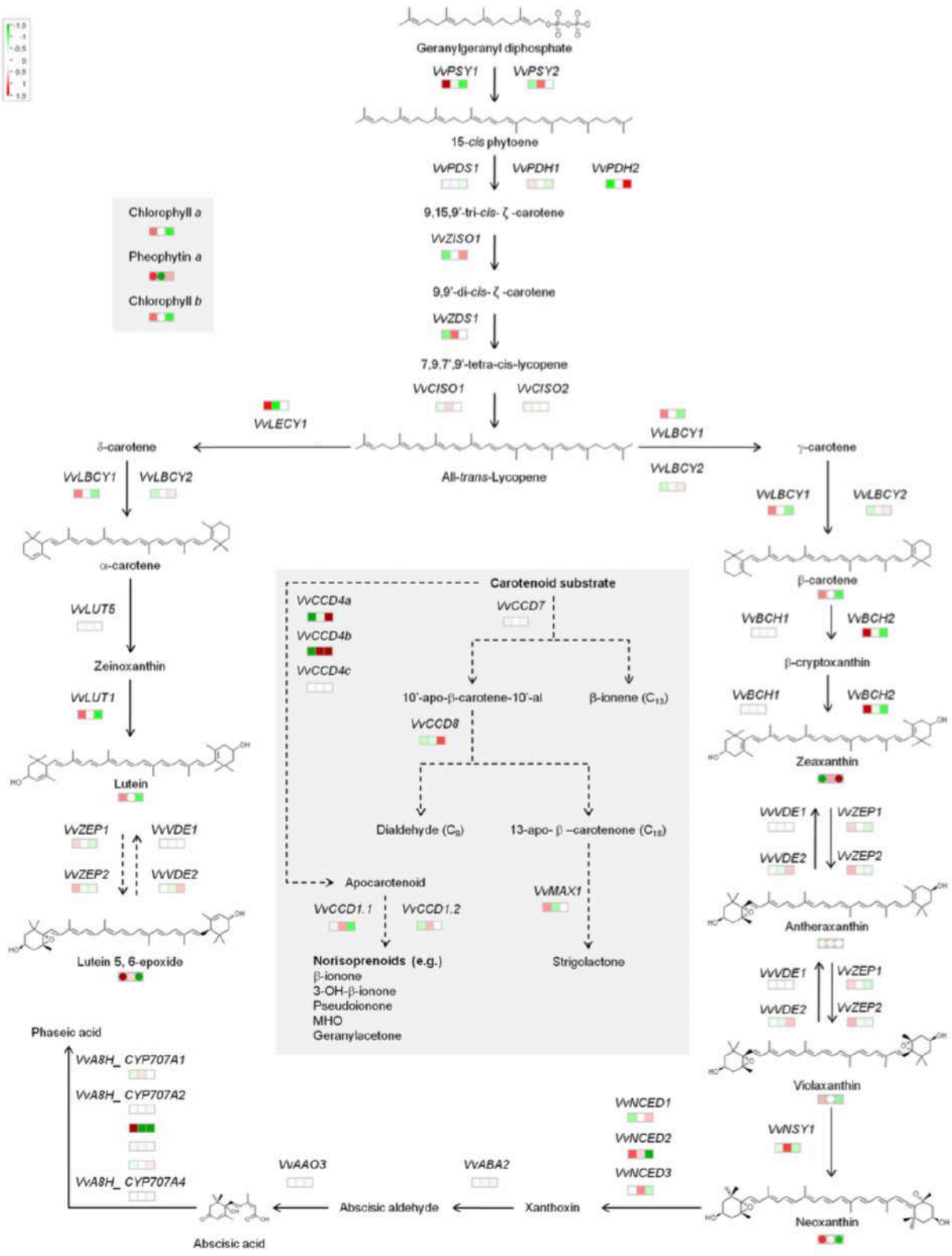

Figure 2.5. The genes and enzymes of the carotenoid biosynthetic and catabolic pathways in grapes (Young et al., 2012). The diagram represents Mapman heat maps of the relative changes in gene and metabolite levels at three berry developmental stages (EL31, EL34 and EL38). The squares indicate $\log_2$ transformed, mean-centered values for genes (squares) and carotenoids (circles).

Although these findings successfully link the effect of a specific viticultural treatment to norisoprenoid accumulation at the end of berry development, our knowledge linking the effects of light exposure on carotenoid accumulation during early grape development remains fragmented.

In 2016, a study addressed this gap in our understanding of the development and degradation of carotenoids in grape berries developing under various light conditions. This publication focused on the accumulation of grape carotenoids and norisoprenoids (and other volatile terpenoid-derived flavor and aroma compounds) in response to various levels of solar light and UV-B exposure to the grape berries (Joubert et al., 2016). In this study, the authors aimed to identify UV-B specific changes in metabolite concentrations in developing grape berries by not only studying the effect of leaf removal, but also the effect of UV-B attenuation through the use of UV-B excluding sheets. The main findings were that photosynthetically active grape berries utilized specific carotenoids and the activation of the xanthophyll cycles to mitigate the effects of UV exposure by acclimating to the environmental stress. Furthermore, the carotenoid-derived norisoprenoid pools were larger in ripe grapes exposed to higher levels of light throughout their development. The authors concluded that the light stress perceived by photosynthesizing berries was successfully mitigated through the activation of the violaxanthin cycle (NPQ) as part of photoprotection. These findings further confirm the notion that green grapes respond to fluctuations in light much like other vegetative/foliar photosynthesizing tissues.

Taken together, these studies provide ample evidence for the indispensable role that carotenoids and apocarotenoids play in berry acclimation to fluctuating levels of light when the grapes are green and photosynthesizing. However, despite the extensive characterization of the genes and enzymes involved in carotenoid/apocarotenoid biosynthesis, few studies have explored the effect that variable levels of light may have on the transcription, regulation and metabolism of carotenoids in grapes and how these compounds are utilized in grape acclimation strategies to mitigate the effects of adverse environmental growing conditions. Future studies are required to elucidate how light may affect carotenoid synthesis on a molecular level and how the degradation of these carotenoids contribute to the aroma profiles of not only the grapes at harvest, but also the wines produced from these grapes exposed to various levels of light.

2.3.1.2 Amino acid metabolism and berry light modulation

As part of amino acid metabolism, several volatile compounds are synthesized that include phenylpropanoids, branched-chain aliphatics and methoxypyrazines (Figure 2.4). The synthesis of phenylpropanoids and flavonoids are arguably the most frequently studied pathways in grapevine research due to their roles in grape responses to abiotic stress and their final contribution to grape quality-related characteristics. A summarized diagram depicting several branch points between primary and secondary grape metabolism towards phenylpropanoid and flavonoid synthesis is presented in Figure 2.6. Among the phenylpropanoids in grapes are hydroxycinnamic acids and flavonoids that all contribute in some way to plant photoprotection. These flavonoid compounds can be further divided into flavonols, favan-3-ols and

anthocyanins. Flavonols, in particular, can not only act as sunscreen molecules themselves, but also have the capacity to quench ROS (Agati et al., 2012; Brunetti et al., 2015). Furthermore, flavonols have been implicated in plant signaling associated with antioxidant homeostasis (Agati et al., 2012).

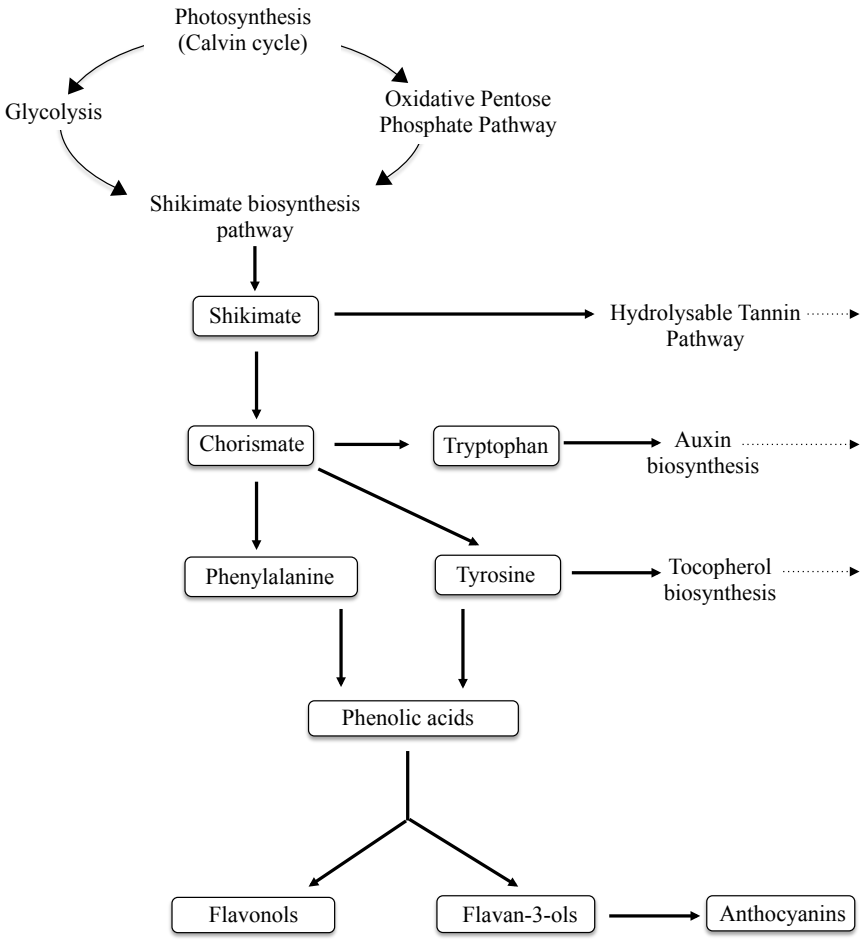

Figure 2.6. A simplified schematic representing the several branch points between primary and secondary metabolism towards the synthesis of phenylpropanoids and flavonoid compounds in grape berries.

The metabolites and enzymes that constitutes this pathway has been studied in response to drought (Corso et al., 2015; Deluc et al., 2009; Król et al., 2014; Savoi et al., 2016), temperature (Azuma et al., 2012; Fernandes De Oliviera et al., 2015; George et al., 2015; Pastore et al., 2017; Rienth et al., 2016), and biotic stress (Blanco-Ulate et al., 2015) by the implementation of several omics technologies. Similarly, the differential metabolic patterns when comparing various grapevine cultivars have also been evaluated (Barros et al., 2014; George et al., 2015; Degu et al., 2014; Giovanelli and Brenna, 2007). It has however been the irrefutable link between light and the regulation of the phenylpropanoid pathway that has been studied most extensively in red (Azuma et al., 2012; Carbonell-Bejerano et al., 2014; Lee and Skinkis, 2013; Matus et al., 2009; Song et al., 2015; Sun et al., 2017; Tardaguila et al., 2010) and in limited white grape cultivars (Joubert et al., 2016). Taken together, these studies confirm that the accumulation of flavonols, anthocyanins and other phenolic compounds increase dramatically from the onset of grape berry ripening (véraison).

These reports further show that their accumulation can be strongly enhanced through elevated exposure to light and that the flavan-3-ols and hydroxycinnamic acids are less prominently affected by light exposure.

The underlying transcriptional mechanisms of flavonoid accumulation in response to light have been characterized as well (Czemmel et al., 2009; Czemmel et al., 2017; Matus et al., 2009). It was established that members of the R2R3-MYB transcription factor superfamily are responsible for the regulation of branches of the flavonoid biosynthesis pathway with great specificity. For example, *VviMYBF1* was found to specifically regulate the expression of several genes involved in flavonol synthesis, including the flavonol synthase encoding gene (*VviFLS1*), whereas *VviMYBA1/2* expression regulates the synthesis of the anthocyanins responsible for red grape skin color (Kobayashi et al., 2004). This view of the phenylpropanoid pathway was recently updated to include novel transcripts encoding enzymes putatively involved in flavonol modification, specifically regulated by *VviMYBF1* in a light and UV-B dependent manner (Czemmel et al., 2017).

Furthermore, a retrotransposon (*Gret1*) in the promotor region of this *VviMYBA1* gene is responsible for the determination of the white grape skin color when present in a homozygous state (Kobayashi et al., 2004). More recently it was reported that large-scale spontaneous genome rearrangements may be responsible for this phenotypic variation in grapevine (Carbonell-Bejerano et al., 2017).

In addition to phenylpropanoid compounds, methoxypyrazines are products of amino acid metabolism in grape berries, synthesized from the downstream metabolism of leucine and isoleucine (Figure 2.4). The irrefutable impact that these methoxypyrazines have on grape and wine aroma was first reported in 1975 by Bayonove et al. Since then, methoxypyrazines have been linked to the accumulation of herbaceous “green” aromas in a select group of *V. vinifera* cultivars including Carmenere (Belancic and Agosin, 2007), Cabernet Sauvignon, its parents, Sauvginon Blanc and Cabernet Franc (De Boubée et al., 2000 Hashizume et al., 2001) and Merlot (reviewed in Sidhu et al., 2015).

Although three methoxypyrazines are readily detected in grape berries, the methoxypyrazine, 3-isobutyl-2-methoxypyrazine (IBMP), is detected at the highest concentrations and are synthesized during the early berry developmental stages after which they are degraded and volatilized during berry ripening (Dunlevy et al., 2013; Hashizume et al., 2001). Previous studies have characterized the likely synthesis pathway involved in IBMP synthesis and have concluded that a group of specific methyltransferase enzymes are responsible for the final methylation step in the synthesis of this aroma compound in grapes (Vallarino et al., 2011). The genes encoding these methyltransferases in grapes were subsequently characterized by Dunlevy et al. (2013).

Reports regarding the impact that light has on the accumulation of methoxypyrazines synthesis in grapes remains contradictory. It has been reported that methoxypyrazine concentrations in grapes depend on

climatic conditions such as light exposure and that the accumulation of IBMP is particularly sensitive to light and temperature (Prouteau et al., 2004). However, the accumulation of IBMP was reported to be unaffected by light in later studies (Liu et al., 2015; Gregan et al., 2012; Šuklje et al., 2014). Therefore, although the genes and enzymes involved in methoxypyrazine synthesis have been characterized, the mechanisms of their degradation remain poorly understood.

Although our knowledge of these amino acid metabolic pathways is currently relatively complete, the metabolic impact that the upregulation of these volatile compounds may have on primary metabolism and development of the grapes remains relatively unclear. Recently, Sun et al., (2017) reported that light induced variations in grape phenolic compounds involved the reprogramming of the whole berry transcriptome, downstream biosynthetic enzymes and hormonal regulators of growth and development. Furthermore, it is well established that the synthesis of flavonoids requires the induction of the shikimate pathway for the synthesis of aromatic amino acids, phenylalanine, tyrosine and tryptophan, to serve as precursors (Figure 2.5). The impact that the upregulation of this energetically costly secondary metabolic process will have on the efficiency of upstream primary metabolic processes remains unexplored.

An insight into the maintenance of primary metabolism may be provided by the amino acid concentrations of the grapes with elevated flavonoid concentrations. In *Arabidopsis*, it was shown that genes encoding amino acid catabolic enzymes were far more sensitive to abiotic stress exposure than those of the enzymes responsible for their synthesis (Less and Galili, 2008). It was also established that the catabolism of these amino acids provides an energetic advantage under stressful environmental conditions (Caldana et al., 2011). In an attempt to elucidate the role that amino acids play in grape flavonoid synthesis, Manela et al., (2015) determined that the aromatic amino acids, phenylalanine and tyrosine, are the rate limiting precursors to flavonoid biosynthesis in grape cell suspensions. These findings provide the first fragmented insights into how grape berries may account for the energetic deficits caused by elevated synthesis of photoprotective flavonoids under high light conditions.

Due to the extensive characterization of the genes, enzymes and metabolites of the phenylpropanoid and flavonoid pathways in grape berries, our understanding of the effect of light exposure on the accumulation of these photoprotective compounds can be successfully manipulated and accurately anticipated through viticultural practices. Our current understanding regarding how grape berries cope with the resource deficit associated with this energetically costly upregulation of flavonoids remains to be explored in more detail and could further our understanding regarding how grape berries acclimate to fluctuating light conditions.

2.3.1.3 Fatty acid metabolism and berry light modulation

Among the grape-derived compounds associated with important impact odorants in wine are the green leaf volatiles (GLVs), appropriately named due to their characteristic contribution to the aroma of freshly cut

grass (Matsui, 2006). These GLVs are synthesized through the catabolism of polyunsaturated fatty acids (PUFAs) as part of lipid metabolism during plant development under normal conditions. Under normal growing conditions, these plant GLVs have been implicated in plant protection against herbivores and it has been reported that the release of these GLVs can by stimulated by wounding (mechanical or herbivory).

The role of these GLVs have however been more frequently studied in the context of abiotic stress resistance in plants. In *Phalaenopsis* plantlets, elevated light exposure induced the synthesis of higher levels of LOX activity (Gayen et al., 2015), similar to the upregulation of *LOX* encoding genes in response to elevated red light exposure reported in *Arabidopsis* (Zhao et al., 2014). An additional function of these enzymes has been reported in several plants systems that implicate these enzymes in the co-oxidation of carotenoids. Aziz et al., (1999) reported the co-oxidation of β-carotene and PUFAs in potato, whereas the downregulation of LOX encoding genes coincided with lower levels of carotenoid degradation in golden rice (Gayen et al., 2015). This co-oxidation of carotenoids by LOXs was extensively reviewed by Chedea and Jisaka (2013).

In grapes, the predominant PUFAs are linoleic and α-linolenic acid that are enzymatically cleaved by lipoxygenase enzymes (LOXs) to form PUFA hydroperoxides that are further rapidly converted by hydroperoxide lyases (HPLs) to form GLVs. HPLs form part of the CYP74 enzyme family with several other members being able to further catabolize PUFA hydroperoxides as well. Some of these CYP74 enzymes include allene oxide synthases (AOS), divinyl ether synthase (DES) and epoxy alcohol synthase (AES) that are responsible for the synthesis of jasmonates, divinyl ether PUFAs and epoxy hydroxyl PUFAs, respectively. These downstream compounds are collectively called oxylipins and fulfill a highly diverse range of physiological functions constitutively or in response to abiotic stresses (for review, see Mosblech et al., 2009). Additionally, some LOX enzymes have the ability to act on PUFA hydroperoxides themselves to synthesize keto PUFAs. The enzymes and genes associated with the lipoxygenase pathway in grapevine were characterized by Podolyan et al., (2010).

Of further particular interest in grape research is the downstream metabolites synthesized from the metabolism of GLVs. These downstream metabolites include a range of non-volatile cysteinylated thiol precursors in grapes that are volatilized by yeast β-lyase activity during alcoholic fermentation as part of the winemaking process (Belda et al., 2016; Swiegers et al., 2009). These volatile thiols and the various combinations and the ratios in which they are present are crucial contributors to wine aroma and quality. Some of the most prominent thiol contributors to wine include 3-sulfanylhexan-1-ol (3SH, formerly known as 3MH) responsible for the grapefruit and passion fruit aromas (Tominaga et al., 1998), 4-methyl-4-sulfanylpentan-2-one (4SMP, formerly known as 4MMP) responsible for the box tree and blackcurrant aromas (Darriet et al., 1995) and 3-sulfanylhexyl acetate (3SHA formerly known as 3MHA) responsible for the box tree aroma (Tominanga et al., 1998).

Due to the potential impact that the LOX-HPL pathway and downstream thiol precursor synthesis may have on the outcome of grapes and wine, recent studies have focused towards factors that may influence these pathways in grapes. Although a recent study reported on the impact that varietal differences have on the LOX-HPL pathway (Qian et al., 2016), most recent studies have focused on the impact that various abiotic stresses, including levels of light may have on LOX activity. Ju et al., (2016) reported on the direct effect that vine leaf removal had on LOX enzyme activity in Pinot noir grapes, whereas Joubert et al., (2016) established that the UV-B component in sunlight is the determining factor affecting GLV accumulation in Sauvignon Blanc grapes developing under high and low solar light exposure. Interestingly, as previously mentioned (section 3.2.1), Joubert et al. (2016) further reported differential accumulation of specific oxygenated carotenoids that further establish the possible co-oxidation activity of LOX enzymes. This co-oxidation of carotenoids by LOX enzymes remains to be further explored in grapevine. A clear understanding of the underlying molecular mechanisms associated with these pathways could allow for more accurate anticipation of the effect of light on the synthesis and development of grape-derived GLVs and volatile thiols.

2.4 Conclusions

The quantity and quality of light that plants are exposed to is ever changing on an hourly, daily and seasonal basis. Understanding the metabolic outcome of these fluctuations are valuable in formulating optimal cultivation practices of economically important crops for the purpose of establishing a repeatable, predictable outcome of specific agronomical manipulations. Through the evolution and integration of several omics technologies, our understanding of how grapes respond on a metabolic and transcriptomic level has evolved as well. Acknowledging the importance of careful planning and executing of vineyard-based experiments where cause-and effect relationships can be established has propelled our understanding of grapevine biology.

Through several recent studies, it is well established that grape berries are able to acclimate to fluctuating light exposure through the implementation of several secondary metabolic strategies in parallel. Some of the secondary metabolic pathways affected by light exposure were found to include isoprenoid, amino acid and fatty acid metabolism, respectively. The metabolites synthesized as products of these pathways all have a potential impact on the quality associated characteristics in grapes and wines and the underlying molecular mechanisms are being systematically uncovered.

Current molecular studies are, however, dependent on the accuracy of the grapevine reference genome (Pinot noir) to represent gene expression of a wide range of genotypically diverse cultivars. The study of the molecular mechanisms involved in these metabolic consequences could be refined to include the *de novo* transcriptome assembly of each specific grapevine cultivars and the construction of a grapevine pan-genome

could contribute to the identification some of the unique transcriptional signatures and potentially unique genotype-dependent responses to environmental modulations such as light.

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Chapter 3

The transcriptional responses and metabolic consequences of acclimation to elevated light exposure in grapevine berries

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The Transcriptional Responses and Metabolic Consequences of Acclimation to Elevated Light Exposure in Grapevine Berries

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An increasing number of field studies that focus on grapevine berry development and ripening implement systems biology approaches; the results are highlighting not only the intricacies of the developmental programming/reprogramming that occurs, but also the complexity of how profoundly the microclimate influences the metabolism of the berry throughout the different stages of development. In a previous study we confirmed that a leaf removal treatment to Sauvignon Blanc grapes, grown in a highly characterized vineyard, primarily affected the level of light exposure to the berries throughout their development. A full transcriptomic analysis of berries from this model vineyard details the underlying molecular responses of the berries in reaction to the exposure and show how the berries acclimated to the imposing light stress. Gene expression involved in the protection of the photosynthetic machinery through rapid protein-turnover and the expression of photoprotective flavonoid compounds were most significantly affected in green berries. Overall, the transcriptome analysis showed that the berries implemented multiple stress-mitigation strategies in parallel and metabolite analysis was used to support the main findings. Combining the transcriptome data and amino acid profiling provided evidence that amino acid catabolism probably contributed to the mitigation of a likely energetic deficit created by the upregulation of (energetically) costly stress defense mechanisms. Furthermore, the rapid turnover of essential proteins involved in the maintenance of primary metabolism and growth in the photosynthetically active grapes appeared to provide precursors for the production of protective secondary metabolites such as apocarotenoids and flavonols in the ripening stages of the berries. Taken together, these results confirmed that the green grape berries responded to light stress much like other vegetative organs and were able to acclimate to the increased exposure, managing their metabolism and energy requirements to sustain the developmental cycle toward ripening. The typical metabolic consequences of leaf removal on grape berries can therefore now be linked to increased light exposure through mechanisms of photoprotection in green berries that leads toward acclimation responses that remain intact until ripening.

Keywords: grape, microclimate, photosynthesis, RNAseq analysis, acclimation to stress

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INTRODUCTION

Plants show remarkable adaptability to environmental factors and/or stresses to ultimately ensure that their core metabolic functions are maintained. Although these aspects have been intensively studied in model plants under controlled conditions to establish the basic principles and underlying pathways, as technologies developed, our ability to study and understand crop plants in their cultivated natural environments are yielding important information regarding the processes of stress protection and specifically the concept of acclimation.

In plant biology, stress is generically defined as any unfavorable conditions that affect metabolism, growth and/or development (Lichtenthaler and Burkart, 1996). The relative tolerance/sensitivity of the affected plant subsequently determines if a stress factor will have a positive (eustress) or negative (distress) outcome (Kranner et al., 2010). Acclimation refers to the short-term responses of plants to adapt to unfavorable (stress) factors in their immediate environment (Lichtenthaler and Burkart, 1996; Lichtenthaler, 1998); whereas adaptation refers to plants' long-term survival strategy to stress factors that occurs via genetic changes such as mutations and subsequent natural selection over many generations within a population. When compared to adaptation, acclimation is a rapid response, occurs within individuals, is reversible, and does not involve any permanent genetic changes. Acclimation can involve transcriptional, metabolic and/or physiological responses to improve the performance and survival of the individual to the stress. The ability of biennial plants (e.g., onions, cabbages, and carrots) to survive winter (Andrews, 1996) and the accumulation of phenolic compounds in response to increased light exposure (Caldwell et al., 1983), are examples of acclimation to low temperature and UV-B, respectively.

In grapevine, acclimation to climatic conditions is particularly important and the plasticity of grapevine responses have been highlighted in a number of publications (overviewed recently in Kuhn et al., 2014). The transcriptomic and metabolic reprogramming occurring during grape berry development has been well studied (Zenoni et al., 2010; Sweetman et al., 2012; Palumbo et al., 2014; Pilati et al., 2014; Wong et al., 2016). Research on abiotic stress factors has focused on the dominant environmental factors either individually: temperature (Carbonell-Bejerano et al., 2013; Rienth et al., 2014), light (Wu et al., 2014; Reshef et al., 2017; Sun et al., 2017), UV (Martinez-Luscher et al., 2014; Suzuki et al., 2015; Matus, 2016), and water deficit (Ghan et al., 2015; Santo et al., 2016; Savoi et al., 2016) or collectively as terroir or vintage studies (e.g., Santo et al., 2013; Anesi et al., 2015).

Light has long been recognized as central to plant metabolism through photosynthesis, but recent studies have highlighted the importance of light as a source of information for plants (reviewed in Apel and Hirt, 2004; Eberhard et al., 2008; Li et al., 2009 and references within). In viticulture, many canopy management practices are performed to optimize light exposure to drive photosynthesis of the canopy (reviewed in Smart, 1985; Clingeleffer, 2010). Apart from leaves, other plant organs including the stems, flowers, tendrils and fruits contain functional chloroplasts, and are capable of photosynthesis (reviewed in Blanke and Lenz, 1989). The conditions under which photosynthesis occurs in these non-foliar organs, however, are markedly different to their foliar counterparts. In fruits, for example, the gradual disappearance of stomata and/or the development of an impermeable waxy cuticle during development results in an internal environment that is characterized by high CO_2 and low O_2 (hypoxic) levels (Blanke and Leyhe, 1987, 1988; Kyzeridou et al., 2015). Decreased photosynthesis in green fruits can be attributed to these physical/anatomical features, rather than a decrease in the photosystems. Kyzeridou et al. (2015) demonstrated that in comparison to leaves, the green fruits of *Nerium oleander* and *Rosa* sp. had higher Car/Chl ratio due to increased xanthophyll cycle components (violaxanthin, antheraxanthin and zeaxanthin) and a lower chlorophyll content. This resulted in a photoprotective xanthophyll cycle that is more functional under high light in green fruits than in leaves. This has also been reported for apple (Cheng and Ma, 2004) and grapevine (Young et al., 2016) and it is speculated that this exists in non-foliar photosynthetic organs to reflect a common strategy for photosynthetic green tissues under similar low oxygen conditions (Kyzeridou et al., 2015).

Some canopy manipulations, such as leaf removal in the fruiting zones are, however, utilized to increase light penetration to the berries (reviewed in Reynolds, 2010). A significant number of studies have investigated the impacts of leaf removal on berry development and ripening. Depending on the cultivar, the objectives range from improving the acid balance (Hunter and Visser, 1990; Toda et al., 2013; Baiano et al., 2015); improving anthocyanin/color stability (Chorti et al., 2010; Sternad Lemut et al., 2011; Lee and Skinkis, 2013; Baiano et al., 2015; Song et al., 2015; Guan et al., 2016; Yu et al., 2016; Pastore et al., 2017); increasing specific secondary metabolites such as volatile aroma precursors (Staff et al., 1997; Tardaguila et al., 2010; Feng et al., 2015; Song et al., 2015; Suklje et al., 2016; Young et al., 2016) or lowering of metabolites that are perceived negatively in the grapes/wines (Sala et al., 2004; reviewed in Sidhu et al., 2015). One of the main outcomes of leaf removal in the bunch zones is the accumulation of protective phenolic compounds i.e., anthocyanins (Lee and Skinkis, 2013; Guan et al., 2016; Lee, 2017) and flavonols (Yu et al., 2016; Pastore et al., 2017), as well as changes to volatile aroma compounds i.e., the norisoprenoid, β-damascenone (Feng et al., 2015; Young et al., 2016) and monoterpenes (Song et al., 2015; Young et al., 2016). These studies have all highlighted the adaptability of the grapevine berries to the changed microclimate and have also provided scope to investigate mechanisms of perceiving and adapting to the stresses linked to changes in microclimate.

Taking advantage of a validated experimental setting where light exposure (to the bunch zone) was the major environmental factor significantly altered by a classic leaf removal treatment in a model Sauvignon Blanc vineyard, the mechanism of berry acclimation to increased light exposure (Young et al., 2016) was targeted in this study. A pertinent result from the phenotyping and metabolite profiling was that none

of the parameters and metabolites measured indicated a compromised primary growth/development and ripening of the berries under the increased exposure. Metabolically, the berries responded to increased light exposure by producing specific secondary metabolites that have photo-protective and/or antioxidant functions. The data generated in the targeted metabolite profiling of the berries lead to the conclusion that the berries mitigated the stress with metabolite reprogramming to acclimate to the increased exposure and that the response was strongly influenced by developmental stage. Although sugars, organic acids, chlorophylls and major photosynthetic pigments (β-carotene and lutein) were not affected by the increased light exposure; specific monoterpenes and photoprotective xanthophylls (zeaxanthin, antheraxanthin, and lutein epoxide) were shown to be increased (Young et al., 2016). These results raised an important question: How were primary metabolism and developmental patterns maintained, despite the light stress-response and metabolic reorganization activated in the exposed berries?

Our primary approach toward achieving these aims was to take a global transcriptional snapshot of gene expression at various berry developmental stages using RNA Sequencing (RNASeq) to thereby create an overview of the effects of elevated light exposure on berry development and ripening. Using this global overview, we were able to target specific metabolic pathways of which gene expression was most significantly affected by the treatment. We could further explore what affects these alterations in gene expression could have on accumulation of metabolites involved in these affected pathways to ultimately determine how berry growth and primary metabolism was maintained despite the activation of stress response mechanisms previously reported (Young et al., 2016).

MATERIALS AND METHODS

Experimental Design, Agronomical Treatments, and Sampling Strategy

The *Vitis vinifera* cv. Sauvignon blanc grapes that were the research materials for this study were harvested from an experimental vineyard located in Elgin region of South Africa during the 2010/2011-harvest season. The complete details pertaining to the climatic measurements, vineyard layout, viticultural practices and sampling strategy of the relevant samples have been performed according to an established field-omics workflow (Alexandersson et al., 2014) and are available in Young et al. (2016). Briefly, grapes were sampled from twelve biological replicates (or panels with six panels per row; and six panels per treatment) in two adjacent vineyard rows (NW-SE row orientation). Each individual biological replicate (panel) consisted of four consecutive vines. The leaf-removal treatment included leaf and lateral shoot removal applied in the bunch zone on the SE-facing side of the canopy at EL29. This leaf-removal treatment was applied to every alternate panel creating a "checkerboard" plot layout where a control panel was always adjacent to an exposed panel (both within a row, and between rows) (Young et al., 2016).

The berries were sampled at green- (pea-sized) (EL31) (Eichhorn and Lorenz, 1977), pre-véraison- (EL33), véraison- (EL35), and the ripe-stage (EL38; corresponding to the commercial harvest date) from control (shaded) and exposed vine panels after which it was frozen in liquid nitrogen in the field. The seeds were removed from the frozen berries in the laboratory and the whole berries, including skins and pulp, were kept at −80°C until subsequent analyses were performed.

Transcriptional Analysis

RNA Extraction and Sequencing

Total RNA was extracted from three out of the six biological replicates sampled at four developmental stages under both exposed and control conditions according to an established protocol (Reid et al., 2006). Each of the 24 samples was subjected to DNAse1 treatment (Sigma-Aldrich, Saint-Louis, MO, USA) to eliminate contamination with genomic DNA. The concentration and purity of the extracted RNA samples were established using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the integrity of the samples were confirmed through analysis of a Bioanalyzer Chip RNA 7500 series II (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions.

After achieving each of the quality control criteria, poly(A) mRNA was prepared for each of the RNA samples and sequenced through an Illumina HiSeq 1000 sequencer according to manufacturing protocols (Illumina Inc., San Diego, CA, USA). The reads generated from the sequencing procedure were aligned to the V1 version of the *V. vinifera* genome (PN40024) using version 2.0 of the TopHat software (Trapnell et al., 2012), allowing a maximum of two nucleotide mismatches. Cufflinks software (version 2.0) was subsequently used in order to assemble transcripts from generated sequence reads (Trapnell et al., 2012), hereby calculating the transcript abundance of each gene in the form of an FPKM value (expected fragments per kilobase of transcript per million fragments mapped). For the purpose of determining which transcripts show differential expression between treatments, CuffDiff (version 2.0) was used after transcript abundances were determined (Trapnell et al., 2012).

RNASeq Expression Data Analysis

When comparing the entire transcriptomes of each of the samples included in this study, Pearson correlations were calculated using R (version 3.3.1) in RStudio (version 0.99.903) and the visualization of the results in the form of a correlation matrix were performed using Microsoft Excel (version 14.1.0).

Gene Ontology (GO) Enrichment analyses of the entire gene lists that showed non-significant differential expression between exposed and control samples at each phenological stage were performed in the BiNGO application in Cytoscape (version 3.4.0) using the Benjamini and Hochberg False Discovery Rate Correction metric. These genes will be further referred to as "unaffected." GO terms were considered significant with a *p*-value smaller than 0.05.

In order to evaluate genes that were significantly affected by elevated light, the results generated from the differential

expression analysis were implemented in a three step process according to different selection criteria. The first step was to perform GO enrichment analysis of all the genes that were significantly differentially expressed ($q \leq 0.05$) under exposed conditions at each developmental stage in order to evaluate the effect that the treatment had on the berry transcriptome throughout development. Next, two distinct thresholds were chosen based upon the number of genes generated that would be most appropriate for subsequent analyses. The first threshold was set to include all differentially expressed genes with a $\log_2$ fold change greater than 1.5 and smaller than −1.5 when comparing the expression of exposed to control genes in order to generate a large list of highly significantly affected genes for the purpose of clustering analysis. This would allow for the identification and evaluation of the most prominent expression profiles of the genes affected by increased exposure without specifically focusing on individual genes. The second threshold was set to include only differentially expressed genes with a $\log_2$ fold change greater than 2 and smaller than −2 for the purpose of focusing on the individual genes that were most affected by increased exposure.

GO enrichment analysis of significantly enriched expression profile clusters of genes expressed at a $\log_2$ fold change ($\log_2$FC) greater than 1.5 between exposed and control grapes during at least one of the phenological stages were performed using the online analysis tool, AgriGO (Du et al., 2010) using the Fisher statistical method with the Yekutieli False Discovery Rate multitest adjustment metric. Significantly enriched GO terms ($p < 0.05$) were further visualized and summarized using the Reduce + Visualize Gene Ontology Web Server (http://revigo.irb.hr; Supek et al., 2011).

For the purpose of performing clustering analysis to infer which genes conform significantly to predetermined gene expression profiles, the Short Time-Course Expression Miner (STEM) was implemented (Ernst et al., 2006). Visualizations of the abovementioned differential expression analyses were performed using Microsoft Excel and Powerpoint (version 14.1.0).

The putative developmental biomarkers were identified and further explored in a three step process. Firstly, the molecular biomarkers of the control grapes representing the two most distinct developmental phases (i.e., green stages vs. ripening stages) were identified by implementing a previously established method (Zamboni et al., 2010). Putative biomarkers that represent the transcriptional difference between the green and the ripening grape berry stages were identified. A two-class OPLS-DA model was generated by representing the expression of green, control berry samples (EL31 and EL33) as its own class as a reference against expression of ripening, control berry samples (EL35 and EL38) set as the second class using SIMCA (version 14.0). An S-plot was subsequently generated to identify the loading correlation coefficient of each gene as described by Zamboni et al. (2010; Wiklund et al., 2008). The aim of this investigation was to generate a broad overview of the developmental progression of the grapes included in this study and therefore, a less stringent correlation cut-off was implemented than in previous studies to identify genes with a loading correlation coefficient higher than 0.8 (positive biomarkers) and lower than −0.8 (negative biomarkers). The expression of positive biomarkers were significantly higher in ripening berries compared to green berries, whereas negative biomarker expression was significantly lower in ripening berries compared to green berries (according to the nomenclature adopted by Zamboni et al., 2010).

Secondly, to establish whether these identified control grape berry developmental biomarkers were comparable to those already established for grape developmental progression, molecular biomarkers identified in this investigation were compared to those published from two previous investigations. The first set of biomarkers included in this comparison was published by Zamboni et al. (2010) in which transcriptional elements unique to early berry development (EL33 and EL35) and late berry development (EL36 and EL38) were identified and named Class a and Class b genes, respectively. These biomarkers will be referred to as early and late developmental markers in subsequent sections of this publication. The second set of genes used to compare the development of the grapes included in this study was published by Palumbo et al. (2014) in which they identified so-called "switch genes" that are considered to characterize the unique transcriptional switch that occurs when grape berries transition from being green, photosynthesizing organs to becoming ripening, sink organs. This aforementioned study utilized transcriptional data generated from five red Italian grape cultivars as well as data generated from the grapevine transcription atlas (Fasoli et al., 2012). A Venn diagram was constructed using the Bioinformatics and Evolutionary Genomics platform (http://bioinformatics.psb.ugent.be/webtools/Venn/) by comparing the genes from the abovementioned studies and the molecular biomarkers identified in this study.

Finally, using the identified developmental biomarkers, the effect of the treatment on the progression of berry development was further explored. This was achieved by determining which of the identified biomarkers shared between this and previous studies were significantly affected by the leaf-removal treatment (and increased exposure) by evaluating the differential expression of these genes.

In order to determine how photosynthesis is affected on a transcriptional level by elevated light exposure, the appropriate gene accessions encoding proteins of PSI and PSII of the thylakoid membranes were obtained from the KEGG Pathway database for *V. vinifera* (http://www.kegg.jp/kegg-bin/highlight_pathway?scale=1.0&map=vvi00195&keyword).

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

In order to validate the accuracy of the gene expression patterns observed in the results generated through RNASeq analysis, RT-PCR was performed using the Applied Biosystems 7500 Real-time PCR System. For these verification assays, total RNA was extracted from three of the six biological replicates originally harvested for metabolic and RNA Seq analyses using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Saint-Louis, MO, USA). cDNA was synthesized from the total RNA using the SensiFAST™ cDNA Synthesis Kit (Bioline, London, UK)

and RT-PCR was performed using the KAPA SYBR®FAST qRT-PCR Kit according to the manufacturer's instructions (Kapa Biosystems, Cape Town, South Africa). Six genes were selected as targets for the PCR reactions based on their expression patterns in response to the treatment as reported by the RNASeq analysis. Four of these target genes were upregulated in response to the treatment by a $\log_2$FC greater than 2 at various developmental stages (VIT_10s0116g00410, VIT_18s0001g03470, VIT_05s0020g04110, VIT_02s0025g04060). The other two of the target genes were related to photosynthesis and were significantly upregulated by elevated light exposure in the green berries (VIT_01s0010g03620, VIT_19s0014g00160). Appropriate primers were designed using QuantPrime (Arvidsson et al., 2008). These primers, their sequences and their characteristics are summarized in **Table S1**. All PCR reactions were performed in triplicate. The normalization and absolute quantification of the expression levels of each of the six genes were performed using the Linear Regression Efficiency (LRE) method using LRE Analyzer software (Rutledge and Stewart, 2008; Rutledge, 2011).

Metabolite Analysis

Extractions and subsequent metabolite analyses were performed from three out of the six available biological repeats that represented the biological triplicates sampled at four developmental stages under both exposed and control conditions.

Amino Acid Analysis

The extraction and HPLC analysis of amino acids in berry samples was performed as described in Antalick et al. (2010), with minor changes. Frozen homogenized berry tissue (200 ± 10 mg) was weighed into 2 mL microfuge tubes and 0.5 mL of 70% (v/v) methanol [containing 25 mg/L of each of the two internal standards (IS), sarcosine and norvaline] was added. Samples were briefly vortexed and sonicated for 10 min at room temperature. After sonication, the samples were centrifuged at 1,250 rpm for 5 min and 200 µL of the supernatant was transferred to amber vials, crimp-sealed and if not analyzed immediately stored at −4°C. Each biological replicate was extracted and analyzed in triplicate. The extracted amino acids were derivatized before analysis on HPLC as described in Suklje et al. (2016).

Major amino acids (AAs) were identified based on their retention times with respect to authentic standard elution and quantified using external standard calibration based on standard curves plotted using the peak areas vs. the standard concentrations. Concentrations were normalized to the IS amount and the sample fresh weight (FW) to obtain the AA concentrations per fresh berry weight (mg/g FW).

Quantification of Phenolic Compound Contents

All authentic standards namely quercetin-glucoside; catechin, epicatechin as well as caftaric acid and caffeic acid as well as the HPLC grade solvents used for sample extraction and separation such as methanol (MeOH, 99.0%), acetonitrile (99.0%), hydrochloric acid (HCl), and the orthophosphoric acid (H_2PO_4, 99.0%) were acquired from Sigma Aldrich (Steinheim, Germany).

Homogenized grapevine berries (200 ± 10 mg) were weighed and 0.5 ml of acidified MeOH (70%; adjusted to pH 1.5 with HCl) was added to each vial, which was then vortexed and sonicated for 15 min at room temperature. After sonication, the samples were centrifuged at 1400 rpm for 5 min and 200 µL of the supernatant was collected and added into amber vials, crimp-sealed for HPLC analysis. Extraction was done in triplicate, in a dark room away from direct light. Extracted flavonoids and phenolic acids in berries were separated and quantified using an Agilent 1100 series HPLC system (Agilent Technologies©, Palo Alto, California, USA) equipped with a diode array detector (DAD) and controlled by a ChemStation Rev. A.10.02 software (Agilent Technologies©). The column used was a Phenomenex Prodigy ODS-2 (4.6 × 150 mm, 5 µm) preloaded with Phenomenex Prodigy guard cartridge (2.1 mm × 100 mm, 1.7 um). The mobile phases were composed of 15% (v/v) H_2PO_4 (A) and 80% acetonitrile containing 20% A (B) and the flow rate was 1 mL/min. The gradient elution conditions started with a linear gradient from 6 to 31% B for 68 min following with another linear gradient from 31 to 65% B for 5 min. Then, the gradient was kept constant at 65% B for 5 min and was decreased from 65%, back to the starting conditions at 6% B for 5 min. The system was re-equilibrated at 6% B for another 10 min before the next injection. The injection volume was set at 20 µL and the column temperature at 40°C.

The major flavonoids and phenolic acids in grapevine berry samples were identified based on their retention times with respect to authentic standard elution and quantified using external standard calibration based on standard curves plotted using the peak areas vs. the standard concentrations. These chromatographic peaks were obtained using the following DAD wavelengths: 280 nm for flavan-3-ols; 360 nm for flavonols and 320 nm for the phenolic acids. Compounds without available standards were quantified using the calibration parameters from quercetin-glucoside (all flavonols) and caftaric acid. The concentrations in samples were normalized to the sample fresh weight (FW) to obtain the sample amount per berry FW (µg/gFW). **Table S2** summarizes the retention time and calibration parameters of all standards used in this analysis.

Lipophilic-Oxygen Radical Absorbance Capacity (L-ORAC) Assay

L-ORAC analysis was performed by the Antioxidant Research Unit (Cape Peninsula University of Technology, South Africa) on three biological replicates (in triplicate) harvested at EL33 and EL38, respectively.

Statistical Analysis

The concentrations generated from the analysis of amino acids and phenolic compounds of the grapes were subjected to multivariate data analysis using Statistica (version 13.0). A repeated measures analysis of variance (ANOVA) was performed to identify the relationship between the increased exposure treatment and the concentrations of the measured compounds (AAs and Phenolic compounds). A Fisher LSD *Post-Hoc* test was conducted for each compound to confirm whether the

concentration of the compound was statistically significantly affected by the treatment (q-value).

Basic statistical analysis of data generated from the L-ORAC assay was conducted in Microsoft Excel (version 14.1.0) using a paired t-test to determine whether exposed grapes had significantly higher lipophilic antioxidant capacity than control grapes at EL33 and EL38.

RESULTS

Overview of the Transcriptional Data Generated

In this study, RNASeq was performed with 24 Sauvignon blanc berry samples representing grapes from shaded (control) and exposed (treatment) microclimates at four developmental stages from a highly characterized vineyard. A summary of the parsed reads from each of the samples and the number of reads that mapped onto the *V. vinifera* cv. Pinot noir reference genome (PN40024) are included in **Table S3**. The complete RNASeq dataset is available in the NCBI's GEO under the series accession, GSE98873.

In order to compare the complete transcriptomes generated for the 24 grape samples, a correlation matrix was generated by implementing a Pearson's correlation coefficient as a distance metric (**Figure S1**). The resulting matrix revealed that one sample harvested at EL38 did not correlate strongly to the rest of the EL38 samples, but rather to samples taken at EL33. Not only were the other 23 samples closely grouped according to their specific developmental stage, targeted metabolite profiling of the same grape samples previously confirmed the close grouping of all the EL38 samples (Figures 3, 4 in Young et al., 2016). This sample was treated as an outlier (anomaly) and excluded from all subsequent analyses.

The Pearson correlation matrix was reconstructed including only the 23 remaining samples and is presented in **Figure 1**; the matrix shows a strong correlation between grapes from the same developmental stage, regardless of the viticultural treatment implemented. Furthermore, gene expression of green berries was more closely correlated between EL31 and EL33 stages than with the two consecutive ripening stages, EL35 and EL38. The correlation matrix also provided confidence in the experimental design and sampling strategy since the biological replicates of the control and exposed treatments confirmed the repeatability of the effect that the leaf removal treatment had on the berry transcriptome at each developmental stage.

Out of the 29,970 genes represented in this version (V1) of the grapevine genome, the expression of 5,050 genes (16.5%) could not be detected across any of the observed developmental stages and treatments and the enriched GO terms representing these genes are summarized as **Figure S2** (as represented by Revigo). A further 4,715 genes with FPKM expression values lower than the recommended reliable RNASeq threshold of an FPKM = 1 (Warden et al., 2013; Massonnet, 2015) throughout all developmental stages and treatments were excluded from further analyses.

RT-PCR analysis of six genes that showed significant upregulation in response to the exposure treatment was conducted and validated the accuracy of the RNASeq results (**Figure S3**). Predominantly, the general expression trend throughout development of each of the genes was similar when comparing the RNASeq and RT-PCR results for control and exposed grapes. These initial analyses not only established confidence in the experimental design and the repeatability among biological replicates, but it further established the accuracy of the RNASeq method and subsequent results generated.

Developmental Biomarker Analysis

In total, the expression of 4,975 genes was identified as developmental phase-specific biomarkers responsible for the greatest transcriptional differences between the green and ripening developmental stages. 2,242 and 2,733 of these genes were positively and negatively correlated (Correlation value $\geq$ 0.8) to the separation, respectively (**Table S4**).

The expression of these markers was comparable to previously established markers for grape berry development (Zamboni

			EL31						EL33						EL35						EL38					
			Exposed			Control			Exposed			Control			Exposed			Control			Exposed			Control		
			Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	
EL31	Exposed	Rep_1	1.00	0.96	0.96	0.96	0.96	0.96	0.94	0.94	0.94	0.94	0.94	0.94	0.91	0.91	0.90	0.91	0.90	0.91	0.86	0.86	0.85	0.86	0.86	
		Rep_2	0.96	1.00	0.97	0.96	0.96	0.96	0.94	0.95	0.95	0.94	0.94	0.94	0.91	0.91	0.90	0.91	0.91	0.91	0.86	0.87	0.85	0.86	0.87	
		Rep_3	0.96	0.97	1.00	0.96	0.97	0.97	0.94	0.95	0.95	0.95	0.95	0.95	0.91	0.92	0.91	0.92	0.91	0.92	0.87	0.87	0.86	0.87	0.87	
	Control	Rep_1	0.96	0.96	0.96	1.00	0.97	0.96	0.93	0.94	0.94	0.94	0.94	0.94	0.90	0.91	0.90	0.91	0.90	0.91	0.86	0.86	0.85	0.86	0.86	
		Rep_2	0.96	0.96	0.97	0.97	1.00	0.97	0.93	0.94	0.94	0.94	0.95	0.94	0.91	0.91	0.90	0.92	0.91	0.91	0.86	0.87	0.85	0.87	0.87	
		Rep_3	0.96	0.96	0.97	0.96	0.97	1.00	0.94	0.94	0.94	0.95	0.95	0.95	0.91	0.91	0.91	0.91	0.91	0.92	0.86	0.86	0.85	0.87	0.87	
EL33	Exposed	Rep_1	0.94	0.94	0.94	0.93	0.93	0.94	1.00	0.96	0.97	0.96	0.96	0.96	0.94	0.95	0.94	0.94	0.94	0.94	0.90	0.90	0.89	0.90	0.90	
		Rep_2	0.94	0.95	0.95	0.94	0.94	0.94	0.96	1.00	0.97	0.96	0.97	0.96	0.94	0.94	0.93	0.94	0.93	0.94	0.90	0.90	0.89	0.90	0.90	
		Rep_3	0.94	0.95	0.95	0.94	0.94	0.94	0.97	0.97	1.00	0.96	0.97	0.96	0.95	0.95	0.95	0.95	0.94	0.95	0.91	0.91	0.90	0.91	0.91	
	Control	Rep_1	0.94	0.94	0.95	0.94	0.94	0.95	0.96	0.96	0.96	1.00	0.97	0.96	0.93	0.94	0.93	0.94	0.93	0.93	0.90	0.89	0.89	0.90	0.90	
		Rep_2	0.94	0.94	0.95	0.94	0.95	0.95	0.96	0.97	0.97	0.97	1.00	0.97	0.93	0.94	0.93	0.94	0.93	0.94	0.90	0.90	0.89	0.90	0.90	
		Rep_3	0.94	0.94	0.95	0.94	0.94	0.95	0.96	0.96	0.96	0.96	0.97	1.00	0.93	0.94	0.93	0.94	0.94	0.94	0.90	0.90	0.89	0.90	0.90	
EL35	Exposed	Rep_1	0.91	0.91	0.91	0.90	0.91	0.91	0.94	0.94	0.95	0.93	0.93	0.93	1.00	0.97	0.96	0.97	0.96	0.97	0.94	0.93	0.93	0.94	0.94	
		Rep_2	0.91	0.91	0.92	0.91	0.91	0.91	0.95	0.94	0.95	0.94	0.94	0.94	0.97	1.00	0.96	0.96	0.95	0.96	0.92	0.92	0.92	0.93	0.92	
		Rep_3	0.90	0.90	0.91	0.90	0.90	0.91	0.94	0.93	0.95	0.93	0.93	0.93	0.96	0.96	1.00	0.96	0.96	0.96	0.93	0.93	0.93	0.93	0.94	
	Control	Rep_1	0.91	0.91	0.92	0.91	0.92	0.91	0.94	0.94	0.95	0.94	0.94	0.94	0.97	0.96	0.96	1.00	0.96	0.97	0.93	0.93	0.92	0.94	0.93	
		Rep_2	0.90	0.91	0.91	0.90	0.91	0.91	0.94	0.93	0.94	0.93	0.93	0.94	0.96	0.95	0.96	0.96	1.00	0.97	0.93	0.93	0.92	0.93	0.93	
		Rep_3	0.91	0.91	0.92	0.91	0.91	0.92	0.94	0.94	0.95	0.93	0.94	0.94	0.97	0.96	0.96	0.97	0.97	1.00	0.93	0.93	0.92	0.93	0.94	
EL38	Exposed	Rep_1	0.86	0.86	0.87	0.86	0.86	0.86	0.90	0.90	0.91	0.90	0.90	0.90	0.94	0.92	0.93	0.93	0.93	0.93	1.00	0.97	0.96	0.96	0.96	
		Rep_2	0.86	0.87	0.87	0.86	0.87	0.86	0.90	0.90	0.91	0.89	0.90	0.90	0.93	0.92	0.93	0.93	0.93	0.93	0.97	1.00	0.97	0.96	0.96	
		Rep_3	0.85	0.85	0.86	0.85	0.85	0.85	0.89	0.89	0.90	0.89	0.89	0.89	0.93	0.92	0.93	0.92	0.92	0.92	0.96	0.97	1.00	0.96	0.96	
	Control	Rep_1	0.86	0.86	0.87	0.86	0.87	0.87	0.90	0.90	0.91	0.90	0.90	0.90	0.94	0.93	0.93	0.94	0.93	0.93	0.96	0.96	0.96	1.00	0.96	
		Rep_2	0.86	0.87	0.87	0.86	0.87	0.87	0.90	0.90	0.91	0.90	0.90	0.90	0.94	0.92	0.94	0.93	0.93	0.94	0.96	0.96	0.96	0.96	1.00	

FIGURE 1 | Pearson correlation matrix of the entire transcriptomes of 23 samples representing exposed and control grapes at four developmental stages (EL31, EL33, EL35, and EL38).

et al., 2010; Palumbo et al., 2014). Furthermore, the expression of 81% of these shared markers developmental markers were not affected by the treatment. The remaining nine genes responsible for the 19% of developmental biomarkers that were affected by the treatment included an auxin-responsive gene (SAUR29; VIT_16s0098g01150), two genes encoding protein subunits of photosystem I and II (VIT_12s0028g01080, VIT_05s0020g03180) and a calmodulin-binding heat shock protein (VIT_14s0006g01030). The results are summarized in **Figure S3**.

Transcriptional Response of the Berries to Increased Exposure

Transcripts That Were Unaffected

The number of annotated genes that were either not expressed, unaffected by the leaf removal treatment or differentially expressed when comparing exposed to control grapes at each of the phenological stages are summarized in **Figure 2**.

GO enrichment analysis of the genes statistically unaffected by the light treatment revealed that GO terms associated with growth and development were enriched throughout development. Among these were GO terms related to "Biosynthetic process," "Signal transduction," "Protein metabolic process," "Translation," "Transport," and "Response to external stimulus" (**Figure 2**). Furthermore, during the developmental stages in which the berries were photosynthetically active and growing in size (EL31, EL33, and EL35), genes associated with the GO terms "Growth" and "Multicellular organismal development" were unaffected by the treatment at EL31 and EL33 as well.

Transcripts That Were Differentially Expressed as a Consequence of the Treatment

By implementing Cuffdiff software, transcripts that were significantly differentially expressed ($q \leq 0.05$) when comparing exposed to control grapes could be identified. For each of the

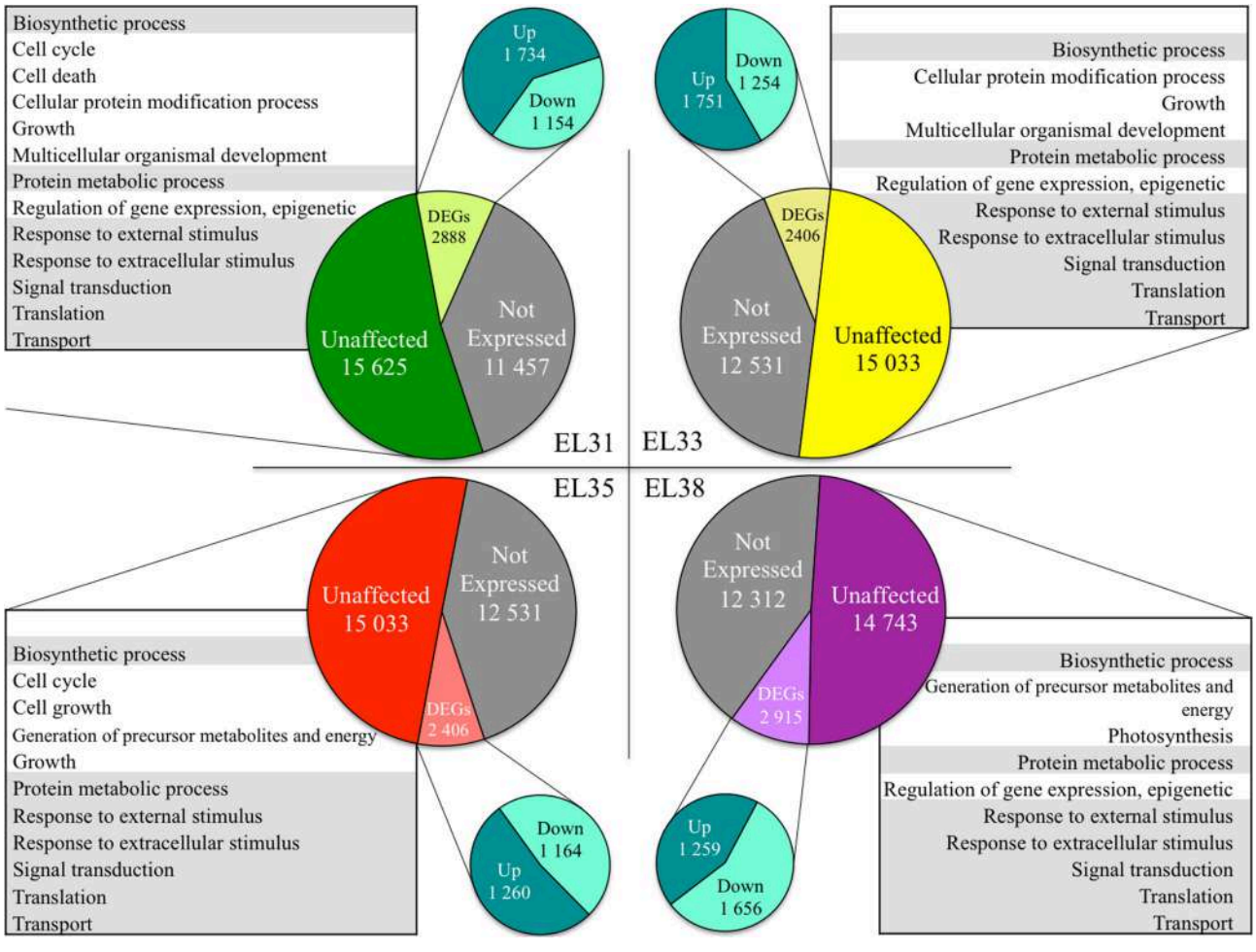


FIGURE 2 | Pie charts representing the number of genes in the grapevine genome either not expressed, significantly unaffected ($q \geq 0.05$) and significantly differentially expressed in response to elevated light ($q \leq 0.05$) at the four phenological stages, respectively. The GO terms significantly enriched representing the genes unaffected by the treatment at each phenological stage are summarized in tables. Gray shading represents GO terms that were commonly unaffected throughout berry development.

four developmental stages being evaluated, the percentage of differentially expressed genes (DEGs) were calculated and the genes that were either significantly up or downregulated in response to the leaf removal treatment could be explored by implementing GO enrichment analysis. The results of these analyses are summarized in **Figure 3**.

These results revealed that grape berries were most significantly affected by the treatment on a transcriptional level during the early developmental stages (EL31 and EL33) and the global description of the biological processes these gene groups were involved with, shared a high degree of similarity between EL31 and EL33 grapes. GO terms associated with photosynthesis and the generation of precursor metabolites and energy were very highly upregulated in exposed grapes until véraison. In the green grapes, especially during EL33, genes associated with the GO terms "cell death" and "response to stress" were among the most significantly downregulated functional groups, exclusively representing genes associated with disease and nematode resistance.

Although ripe berries had the highest number of DEGs in response to the treatment, the enrichment of the GO terms affected by the treatment were lower in comparison to the preceding developmental stages. These enriched GO terms were further associated with genes that were significantly downregulated in response to the treatment as opposed to the preceding stages that were dominated by upregulation in response to increased exposure.

Out of the 29970 genes included in the grapevine genome, 723 genes showed either significant up or downregulation with a factor greater than 1.5 ($\log_2$FC) during at least one developmental stage in response to the elevated light treatment. Clustering analysis revealed that the expression of 431 of these genes could be grouped to seven expression profile clusters as predetermined by the STEM software (**Figure 4A**), with the GO subcategories provided in **Figure 4B** and the genes within each cluster summarized in **Table S6**.

Two of these identified expression clusters (clusters 1 and 2) were represented by genes that followed the predicted developmental progression whilst simultaneously being affected by the treatment. Cluster 1 ($p = 5E^{-90}$; total of 128 genes) represented genes that were simultaneously

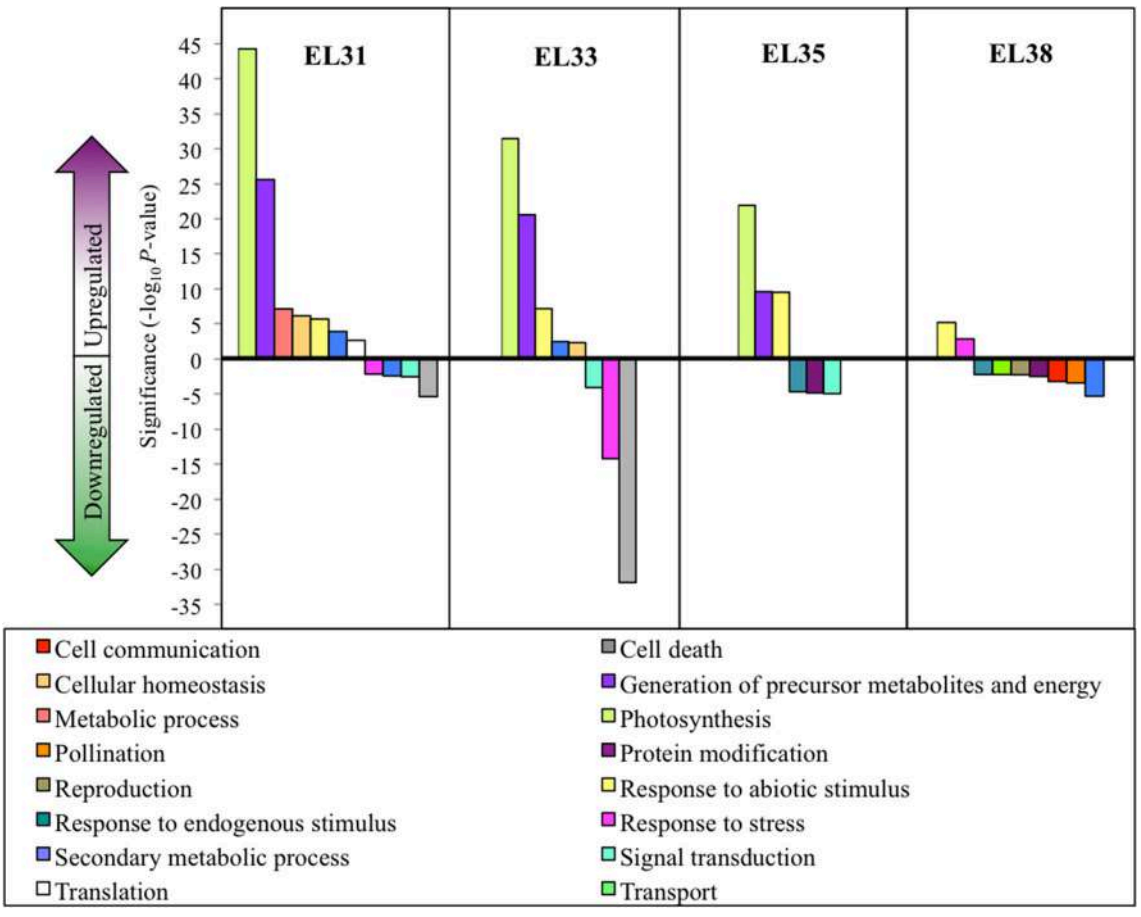


FIGURE 3 | Grape berry transcripts that are significantly differentially expressed in response to elevated light exposure at four phenological stages. Significantly enriched GO categories ($q \leq 0.05$) at each phenological stage. Significance is represented as $\log_{10}$ *P*-values of each GO category with positive values indicating upregulation and negative values indicating downregulation.

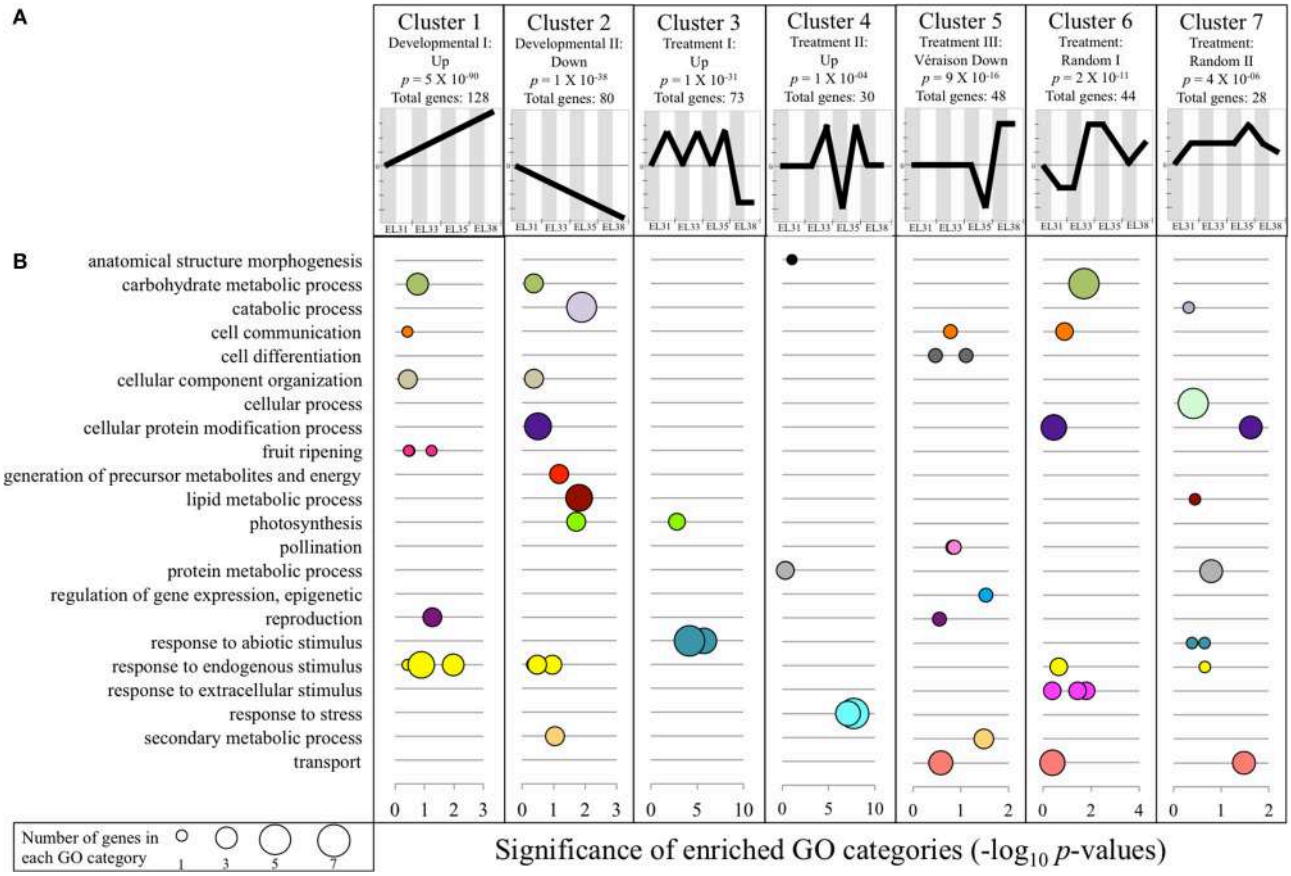


FIGURE 4 | Expression clustering analysis results of all genes differentially regulated ($q \leq 0.05$) by a $\log_2$ fold-change higher by a factor of 1.5 ($1.5 \leq \log_2 FC \leq -1.5$) in response to elevated light exposure during at least one of the four phenological stages. **(A)** The seven expression profiles to which a significant amount of genes aligned. Shaded columns (gray) indicate the expression of the genes in that specific cluster under control conditions and white columns indicate expression of the same genes under exposed conditions at the developmental stage indicated in the X-axis below. Significance is indicated in each cluster profile representation in the form of a *P*-value. **(B)** Functional GO subcategories of each significantly enriched expression cluster summarized within representative GO terms as summarized by ReviGO. Significance is represented as $-\log_{10}$ *P*-values of each subcategory; the size of each data circle indicates the number of genes that is represented within each enriched GO term.

driven by the increased exposure treatment as well as developmental cues. Several of the functional annotations were associated with the progression of grape berry development, but also secondary metabolic processes linked to abiotic stress responses. Examples of genes within cluster 1 included three Ethylene-responsive transcription factors (VIT_07S0031G01980, VIT_01S0150G00120, VIT_14S0108G00050), a 2-oxoglutarate/malate carrier protein (UCP5; VIT_18S0001G07320) that has been proposed to be involved with acid regulation in grape berries (Chen et al., 2015), a Galactinol synthase (GolS4; VIT_01S0127G00470) involved in the synthesis of the osmoprotectant oligosaccharide, raffinose, a gene encoding a Gamma-aminobutrytic acid transporter (VIT_13S0074G00570), two genes encoding enzymes involved in the phenylpropanoid/flavonoid pathway (anthocyanidin 3-O-glucosyltransferase, VIT_12S0034G00130; Flavanone 3-hydroxylase, VIT_16S0098G00860), as well as the early light-inducible protein (ELIP1, VIT_05S0020G04110) involved in the inhibition of chlorophyll biosynthesis.

Interestingly, 64 of the genes represented by cluster 1 were also identified as developmental biomarkers (**Figure S4**) of which five were shared with the analyses of Zamboni et al. (2010) and Palumbo et al. (2014). One of these genes is a 9-cis-epoxycarotenoid dioxygenase encoding gene (NCED; VIT_02S0087G00930) responsible for the degradation of carotenoids synthesized during the early developmental phases to produce the plant hormone, abscisic acid (ABA) that further plays a pivotal role in plant adaptation to stress.

Cluster 2 represented 80 genes that showed significant downregulation throughout development under exposed conditions, while simultaneously following the same developmental progression. Among the GO terms associated with this cluster were "lipid metabolic process" that represented two senescence-associated genes (SAG101, VIT_14S0066G01830, VIT_14S0066G01820) involved in stress-related signaling, as well as the GO terms "photosynthesis" and "generation of precursor metabolites and energy" that both represented genes that encode a photosystem II PsbO

protein (VIT_18S0001G11710), an LHB1B1 light harvesting protein (VIT_12S0028G00320) and another a polyphenol oxidase chloroplast precursor (VIT_10S0116G00560). Cluster 2 also contained an Alanine-glyoxylate aminotransferase encoding gene (Alanine-glyoxylate aminotransferase 2 3, mitochondrial, VIT_08S0058G00930) that plays a central role in the photorespiratory pathway and a gene encoding a trehalose-6-phosphate phosphatase (VIT_00S0304G00080) that is known to have an indispensible role in normal plant growth and development. Furthermore, 18 of the genes represented by cluster 2 have been identified as negative biomarkers in this study (**Figure S4**).

Clusters 3 and 4 (**Figure 4**) contained genes that were highly responsive to the elevated light exposure treatment regardless of the developmental profile. The genes represented by these clusters show strong functional associations to the activation of several protection mechanisms of the photosynthetic machinery, activated at either the first (EL31) or the second green developmental stage (EL33). Several heat shock protein (HSP) encoding genes, including the well-known abiotic stress signaling regulator, heat shock factor 2A (VIT_04S0008G01110), alongside its putative co-activator, Multiprotein-bridging factor 1 (VIT_11S0016G04080), as well as small HSPs formed part of these clusters.

Cluster 3 further represented several genes that contributed to the GO term, "photosynthesis." These included a gene encoding a chloroplastic carbonic anhydrase (VIT_14S0066G01210) critical in the maintenance of the rate of photosynthetic CO_2 fixation, and a photosystem II protein encoding gene (PsbP, VIT_13S0019G00320) that forms part of the oxygen evolving complex of PSII, specifically contributing toward its stabilization. Furthermore, a WUSCHEL encoding gene (VIT_18S0001G10160) was present in this cluster that represents a member of a transcription factor gene family involved in reproductive organ development, hormone signaling and abiotic stress response in several plant species.

The 30 genes represented by expression cluster 4 show significantly higher expression from EL33 until véraison after which the expression of these genes was unaffected in ripe berries in response to the treatment. Among the 24 genes within this cluster that had been functionally annotated, an FtsH protease encoding gene (VIT_14S0108G00590), known to be involved in the efficient turnover of the D1 protein of PSII in response to photooxidation, as well as a Calmodulin encoding gene (VIT_18S0122G00180) known to be involved in stress perception and signaling related to cellular calcium ion (Ca^{2+}) concentration in plants were included. Furthermore, this cluster represented genes encoding a galactinol synthase (VIT_07S0005G01970), a Methyl jasmonate esterase (VIT_00S0253G00150) and a 2-oxoglutarate-dependent dioxygenase (VIT_05S0049G00220) among others. Clusters 3 and 4 therefore point toward the activation and maintenance of light stress mitigation strategies during the green developmental stages.

The remaining three clusters (clusters 5, 6, and 7) represented genes that were differentially affected by elevated light exposure according to neither a unique developmental pattern nor consistently by the treatment (**Figure 4**; **Table S6**). Due to the random and complex nature of their transcriptional responses, these gene clusters were not further investigated for the purpose of this study.

In the second step taken to elucidate which transcriptional elements are the most significantly affected by elevated light exposure at each individual stage, genes that show a Log_2 fold change (Log_2FC) either higher than 2 or lower than −2 in exposed compared to control grapes were further explored. In total, 245 and 157 genes were up and downregulated in exposed compared to control grapes according to these criteria, respectively. These genes are listed in **Table S5** and their functional associations are summarized in **Figure 5**.

Among these 245 significantly upregulated genes, 185 were uniquely upregulated at very high levels at each developmental stage investigated with 12, 47, 61, and 65 genes upregulated ($Log_2FC \geq 2$) at EL31, EL33, EL35, and EL38, respectively. Out of the 157 genes that were most significantly downregulated ($Log_2FC \leq -2$), 156 of these genes were uniquely downregulated at either EL31 (28 genes), EL33 (29 genes), EL35 (12), or EL38 (87) in response to elevated light exposure. Several genes were similarly upregulated in various developmental stages (**Figure 5**). These genes, their functional annotations and the significance of their differential expression (q-values) are summarized in **Table S7**.

Metabolic Processes Most Affected by Elevated Light Exposure

The global transcriptional analysis of Sauvignon blanc grape berries yielded insights into which metabolic processes are most affected by elevated light exposure. Gene expression involved in photosynthesis and the synthesis of flavonoid compounds were most significantly activated by the light treatment, which warranted further investigation into how subsequent primary and secondary metabolism of the grape berries was affected by the treatment. In order to investigate these metabolic processes, the synthesis and degradation of the amino acid transcription and composition was further investigated and explored in the context of how this AA metabolism may affect secondary metabolism in response to elevated light exposure in the berry bunch zone.

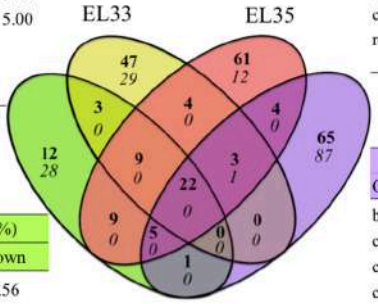
Protection of the Photosynthetic Machinery

The 24 genes included in the investigation of PSI and PSII, their functional annotations and the Log_2 fold change of the expression of each gene when comparing exposed to control grapes at each developmental stage is summarized in **Figure 6**. Every gene included in this analysis was significantly upregulated ($q \leq 0.05$) in response to the leaf removal treatment at EL31.

Similarly, during EL33 and EL35, most of the genes of PSI and PSII remained significantly upregulated with the exception of one LHCA gene (LHCA5, VIT_18s0001g10550), two Psb encoding genes (PsbP, VIT_13s0019g00320; PsbZ, VIT_12s0059g01810) that were unaffected from véraison onwards and a PsbQ (VIT_19s0014g05080) that was unaffected by elevated light exposure from EL33 onwards. Thereafter, at EL38, with the exception of one CAB encoding gene (LHCII type I CAB-1, VIT_19s0014g00160), all of the genes evaluated became either

EL33	Percentage (%)	
GO description	Up	Down
carbohydrate metabolic process		5.00
catabolic process		5.00
cellular component organization		5.00
cellular protein modification process	16.67	5.00
flower development		20.00
fruit ripening	16.67	
generation of precursor metabolites and energy		5.00
reproduction	16.67	5.00
response to endogenous stimulus	33.33	
transport	16.67	

EL35	Percentage (%)	
GO description	Up	Down
signal transduction		33.33
metabolic process		66.67
reproduction	5.00	
generation of precursor metabolites and energy	5.00	
transport	15.00	
response to abiotic stimulus	25.00	
post-embryonic development	25.00	
carbohydrate metabolic process	10.00	
cellular homeostasis	5.00	
response to endogenous stimulus	10.00	



EL31	Percentage (%)	
GO description	Up	Down
carbohydrate metabolic process	14.29	5.56
catabolic process	28.57	11.11
cell differentiation		16.67
cellular component organization		5.56
generation of precursor metabolites and energy	14.29	
lipid metabolic process	14.29	
metabolic process		38.89
response to abiotic stimulus	28.57	
secondary metabolic process		11.11
transport		11.11

EL38	Percentage (%)	
GO description	Up	Down
biosynthetic process		8.70
carbohydrate metabolic process		10.87
catabolic process		6.52
cell communication		2.17
cellular component organization		4.35
cellular protein modification process		8.70
embryo development	35.71	
metabolic process		41.30
pollen-pistil interaction		4.35
reproduction	7.14	2.17
response to biotic stimulus		2.17
response to endogenous stimulus	21.43	2.17
secondary metabolic process	7.14	6.52
transport	28.57	

FIGURE 5 | Venn diagram summarizing the functional associations of the genes up or downregulated by a Log_2 fold-change greater than 2 and smaller than −2 when comparing exposed to control grapes at four phenological stages. The number of upregulated genes is indicated in bold and the number of downregulated genes is italicized in the Venn diagram and GO annotations of the genes uniquely highly differentially regulated at each developmental stage. GO descriptions indicate GO terms that were representative of each gene group, percentages indicate the percentage of genes that are represented by each GO description.

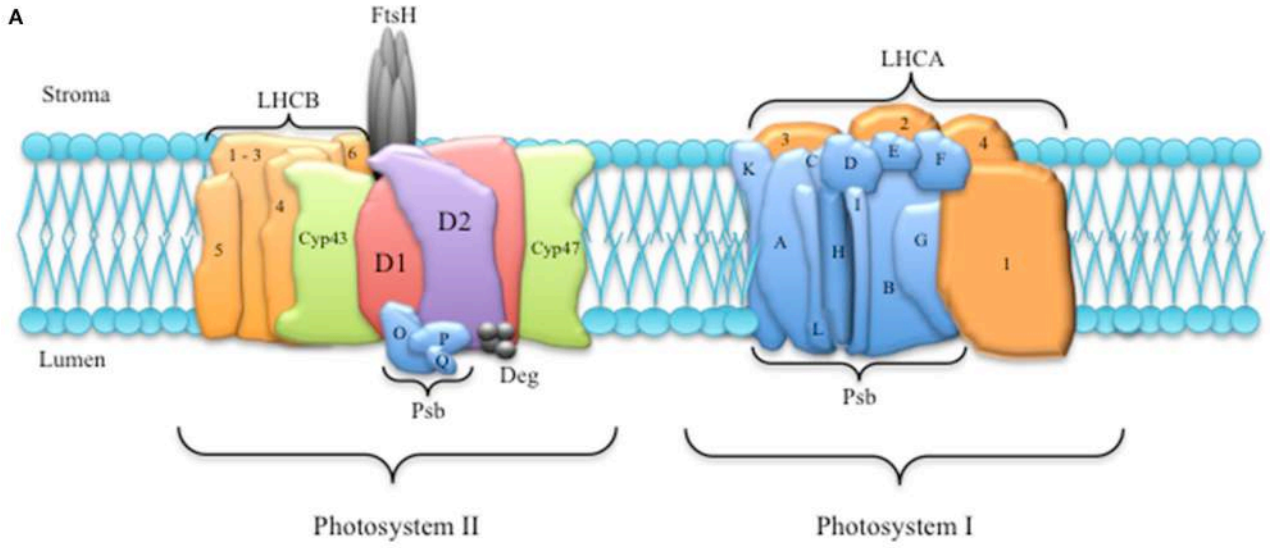
unaffected by the treatment or significantly downregulated in response to the treatment.

The genes putatively encoding enzymes involved in photoprotection mechanisms in grapevine have been acquired from *Arabidopsis* orthologs and the log_2FC of their expression when comparing exposed to control grapes at each developmental stage and is also summarized in **Figure 6**. At EL31, all the genes encoding the enzymes of both non-photochemical quenching (NPQ) and reversible photoinhibition were significantly upregulated with exception of one FtsH protease-encoding gene (VIT_14S0108G00590). Although the abovementioned FtsH protease appeared to be highly upregulated ($Log_2FC = 7.32$), it's expression proved to be highly variable among the biological replicates in this study and was therefore not significantly different when comparing exposed to control berries at EL31. The genes encoding NPQ associated proteins that include PsbS (VIT_18s0001g02740) and one violaxanthin deepoxidase enzyme (VDE) encoding gene (VIT_04s0043g01010) were strongly upregulated by the treatment at EL31. At EL33, however, the FtsH protease-encoding gene (VIT_14S0108G00590), putatively responsible for the degradation of damaged copies of the D1 protein, was most significantly and highly upregulated until the berries were ripe (EL38).

The Effect of Elevated Light Exposure on Amino Acid Metabolism of Developing Grape Berries

HPLC analysis was performed that yielded the concentrations of 23 amino acids at the four developmental stages. The amino acid (AA) concentrations generated for each of the samples generated are included in **Table S8**. The log_2FC values and statistical significance between exposed and control grapes are summarized in **Table 1**. Among these 23 amino acids, the concentrations of eight of these were not affected by the leaf removal treatment at any of the developmental stages evaluated. The only amino acid that was affected by the leaf removal treatment throughout the entire berry development was Gly that was present at significantly higher concentrations from EL31 until EL38.

Taken together these results revealed that, with the exception of Gly, most of the AA concentrations remained unaffected by the treatment until the onset of ripening, followed by the accumulation of significantly altered AA concentrations when comparing exposed to control grapes.



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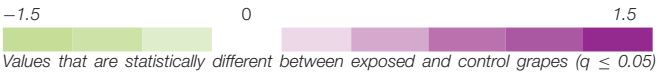
	Functional annotation	Gene accession	Log2 Fold change EL31	EL33	EL35	EL38
Photosystem I	LHCA1	*VIT_13s0019g04140*	**0.96**	**1.11**	**1.06**	-0.02
	LHCA2 (Photosystem I light harvesting complex gene 2)	*VIT_01s0010g03620*	**0.90**	**0.75**	**0.62**	-0.30
	LHCA3 (Photosystem I light harvesting complex gene 3)	*VIT_15s0024g00040*	**0.72**	**0.62**	**0.74**	-0.32
	LHCA4 (Photosystem I light harvesting complex gene 4)	*VIT_17s0000g06350*	**0.87**	**0.92**	**1.33**	-0.05
	LHCA5 (Photosystem I light harvesting complex gene 5)	*VIT_18s0001g10550*	**0.81**	0.27	0.32	-0.71
	LHCA6 (Photosystem I light harvesting complex gene 6)	*VIT_11s0016g00730*	**1.36**	**0.78**	**0.87**	**-1.35**
Photosystem II	LHB1B1	*VIT_12s0028g00320*	**1.52**	**1.36**	**1.29**	**0.82**
	LHCB2.1 (Photosystem II light harvesting complex gene 2.1)	*VIT_12s0057g00630*	**0.63**	**0.49**	**0.48**	-0.24
	LHCB3 (LIGHT-HARVESTING CHLOROPHYLL BINDING PROTEIN 3)	*VIT_00s0181g00180*	**0.98**	**1.09**	**1.40**	0.30
	LHCB4 chlorophyll A-B binding protein CP29	*VIT_08s0007g02190*	**0.76**	**1.00**	**0.71**	-0.26
	LHCB5 (LIGHT HARVESTING COMPLEX OF PHOTOSYSTEM II 5)	*VIT_18s0089g01170*	**0.89**	**0.84**	**0.72**	-0.30
	LHCB6 (LIGHT HARVESTING COMPLEX PSII)	*VIT_12s0055g01110*	**1.01**	**1.20**	**1.07**	-0.03
	LHCII type I CAB-1	*VIT_07s0005g02220*	**0.70**	**0.62**	**1.09**	-0.07
	LHCII type I CAB-1	*VIT_10s0003g02890*	**0.66**	**0.94**	**1.07**	-0.21
	LHCII type I CAB-1	*VIT_10s0003g02900*	**0.68**	**1.08**	**1.31**	-0.67
	LHCII type I CAB-1	*VIT_19s0014g00160*	**1.04**	**0.46**	**0.71**	**0.96**
	photosystem II protein P PsbP	*VIT_13s0019g00320*	**1.84**	**1.14**	0.57	-0.31
	photosystem II PsbO protein	*VIT_00s0207g00210*	**0.62**	**0.58**	**0.33**	-0.18
	photosystem II PsbO protein	*VIT_18s0001g11710*	**1.01**	**1.32**	**1.59**	-0.45
	photosystem II psbZ	*VIT_12s0059g01810*	**1.04**	**1.21**	0.26	**-0.56**
	photosystem II oxygen-evolving enhancer protein PSBQ	*VIT_00s0904g00010*	**0.77**	**0.58**	**0.61**	**-0.54**
	oxygen evolving enhancer 3 (PsbQ)	*VIT_19s0014g05080*	**1.11**	0.48	0.41	**-0.69**
	photosystem II reaction centre W (PsbW)	*VIT_07s0005g04400*	**0.99**	**0.87**	**0.80**	-0.03
	LHCII type III, chloroplast precursor	*VIT_19s0014g03660*	**0.82**	**1.01**	**1.23**	-0.11
Non-photochemical quenching	photosystem II 22 kDa protein PSBS	*VIT_18s0001g02740*	**1.05**	**1.13**	**1.02**	**0.68**
	violaxanthin de-epoxidase	*VIT_04s0043g01010*	**1.26**	**0.57**	**0.49**	0.24
	violaxanthin de-epoxidase	*VIT_07s0031g01770*	**0.82**	0.34	0.29	0.41
Reversible photoinhibition	serine/threonine-protein kinase SNT7, chloroplast precursor	*VIT_08s0058g01450*	**0.76**	**0.62**	0.10	0.20
	serine/threonine-protein kinase SNT8, chloroplast precursor	*VIT_01s0011g03010*	**0.92**	**0.45**	0.14	0.28
	Ftsh protease	*VIT_14S0108G00590*	7.32	**3.73**	**6.36**	**2.21**
	FtsH protease (VAR2)	*VIT_12s0028g01600*	**0.93**	**0.36**	**0.39**	0.27
	DegP protease	*VIT_18s0072g00970*	**0.41**	**0.39**	**0.41**	**-0.45**
	PsbA	*VIT_13s0019g02630*	**0.56**	**0.87**	0.32	0.01

FIGURE 6 | Grape berry photosynthesis and mechanisms of photoprotection. **(A)** A Simplified schematic model of the photosynthetic machinery located in the thylakoid membrane within the chloroplasts. **(B)** A table of representative candidate genes involved in photosystem I and II and two mechanisms of photoprotection in the form of non-photochemical quenching and reversible photoinhibition (RPI), their accessions and the $\log_2$ fold-change when comparing their expression levels (FPKM) between exposed and control grapes at each developmental stage. Significant differences in expression between exposed and control grapes are indicated in bold.

At véraison (EL35) 10 out of the 23 AAs measured were present at significantly lower concentrations in exposed grapes, including the four key nitrogen assimilation AAs, Asp, Asn, Glu, Gln, as well as Ala, Arg, Cys, Met and two aromatic AAs, Phe, and Trp. When the berries achieved ripeness at EL38, GABA, Met, Pro, and Val were present at significantly higher concentrations

TABLE 1 | The fold change (Log_2) of the amino acid concentrations (mg/gFW) of developing grapes when comparing exposed to control berries at four phenological stages.

	Exposed vs. Control (Log_2 fold change)			
	EL31	**EL33**	**EL35**	**EL38**
Ala	0.26	0.17	−0.29	0.02
Arg	−0.78	0.04	−0.50	−0.38
Asn	0.00	−0.99	−1.04	−1.08
Asp	−1.12	−0.53	−0.87	−1.05
Cys	0.00	−0.28	−1.06	−0.04
Cys-Cys	0.59	−0.06	0.01	0.39
GABA	0.26	0.14	0.39	0.63
Gln	−0.28	−0.82	−0.81	−0.80
Glu	−0.10	−0.14	−0.77	−0.01
Gly	1.55	1.15	0.98	0.31
His	−0.50	−0.11	−0.18	−0.19
Ile	0.00	2.13	−0.37	0.01
Leu	−0.77	0.16	−0.23	0.10
Lys	0.00	1.64	0.40	−0.03
Met	0.45	−0.28	−0.87	0.30
Orn	1.21	0.39	0.48	−0.02
Phe	−0.97	0.15	−0.68	−0.41
Pro	0.66	0.44	0.53	0.30
Ser	0.31	−0.26	−0.07	−0.20
Thr	−0.32	0.12	−0.02	−0.10
Trp	−0.20	−0.39	−0.64	−0.99
Tyr	−0.39	0.13	−0.26	0.01
Val	−0.18	0.56	−0.21	0.22
Tot AA	−0.23	−0.48	−0.56	−0.22



Values that are statistically different between exposed and control grapes ($q \leq 0.05$) are colored according to either higher or lower concentrations. These colors indicate higher or lower log_2 fold changes between exposed and control grapes based on their concentrations but are not indicative of higher or lower concentrations themselves.

along with Gly whereas Arg, Asp, Phe, and Trp remained present at lower concentrations in exposed grapes. At this stage, His concentrations were also significantly lower when comparing exposed to control grapes.

To explore the transcriptional regulation of the synthesis and degradation of several of the AAs that were present at altered concentrations in response to the leaf removal treatment, four metabolic pathways including several of the altered AAs were targeted for further investigation. These metabolic pathways included Gly metabolism (**Figure 7A**), the superpathway of Lys, Met and Thr metabolism (**Figure 7B**), the superpathway of Trp, Phe, and Tyr metabolism (**Figure 7C**) and the pathway that involved Pro, Arg, and GABA metabolism (**Figure 7D**). The genes putatively involved in these metabolic pathways according to the current available gene annotation collection are indicated by numbers in the appropriate diagrams and are summarized in **Table S9**.

By evaluating these four AA metabolic pathways it became clear that transcription of the biosynthetic enzyme encoding genes were only marginally affected by the leaf removal treatment, whereas the genes encoding enzymes responsible for the degradation of many of the evaluated AAs were transcriptionally far more reactive to the treatment in the ripening period. The pathway depicting Trp, Phe, and Tyr metabolism (**Figure 7C**) is one example of this upregulation of AA catabolic enzyme encoding genes where the genes responsible for the synthesis of Phe and Tyr were not significantly affected by elevated light exposure at any of the berry developmental stages. The Phe ammonia lyase (PAL) encoding genes (VIT_06s0004g02620, VIT_08s0040g01710, VIT_13s0019g04460) and the Tyr aminotransferase encoding genes (VIT_00s0225g00230, VIT_00s0394g00040) respectively responsible for the degradation of Phe and Tyr were, however, significantly differentially expressed in response to the increased exposure at various stages of berry development.

Increased exposure had distinctly different consequences on grape AA metabolism when comparing green to ripening berries. An example of this developmental, stage-specific metabolism was evident in the upregulation of AA catabolic enzymes in the pathways involved in Gly synthesis (**Figure 7A**) whereby Gly synthesis from the catabolism of both Ser and Glyoxylate were higher in exposed grapes during the green berry stages under elevated light conditions. Conversely, during the berry ripening stages, the synthesis of Gly from the degradation of Ser and Thr by the upregulation of catabolic enzyme encoding genes were higher in exposed grapes. The degradation of several of these AAs will make their constituents, whether secondary compounds or other AAs, available as substrates to secondary metabolic processes that warranted further investigation.

Metabolic Shifts between Primary and Secondary Metabolism in Response to Elevated Light Exposure throughout Berry Development

For the purpose of determining how elevated light exposure could shift developing grape primary and secondary metabolism, a summarized diagram was constructed to evaluate several metabolic branch points by integrating transcriptomic and metabolomic data generated from the same developing grape berries (**Figure 8**). The diagram overlays the concentrations of AAs, phenolic acids and flavonoid compounds in developing grapes with the expression levels of the transcripts known to be responsible for the enzymatic steps in the metabolic pathway between primary and secondary metabolism (**Table S10**). This integrated metabolic pathway focused on the branch point at which Shikimic acid could be either utilized toward the synthesis of hydrolysable tannins or toward the synthesis of chorismate, which serves as substrate for multiple downstream metabolic processes that include the synthesis of auxin from Trp or the synthesis of Tyr or Phe. Tyr in turn serves as a substrate for either the synthesis of the lipophilic antioxidants, tocopherol, or the synthesis of hydroxycinnamic acids from tyramine. Phe on the other hand is an aromatic AA that serves as a precursor for the synthesis of several secondary metabolites such as phenolic acids and flavonoid compounds that could serve as antioxidant molecules under abiotic stress conditions.

The synthesis of higher levels of hydroxycinnamic acids in green grapes were facilitated by both the upregulation of genes encoding the catabolism enzymes of Tyr (VIT_07s0005g04480,

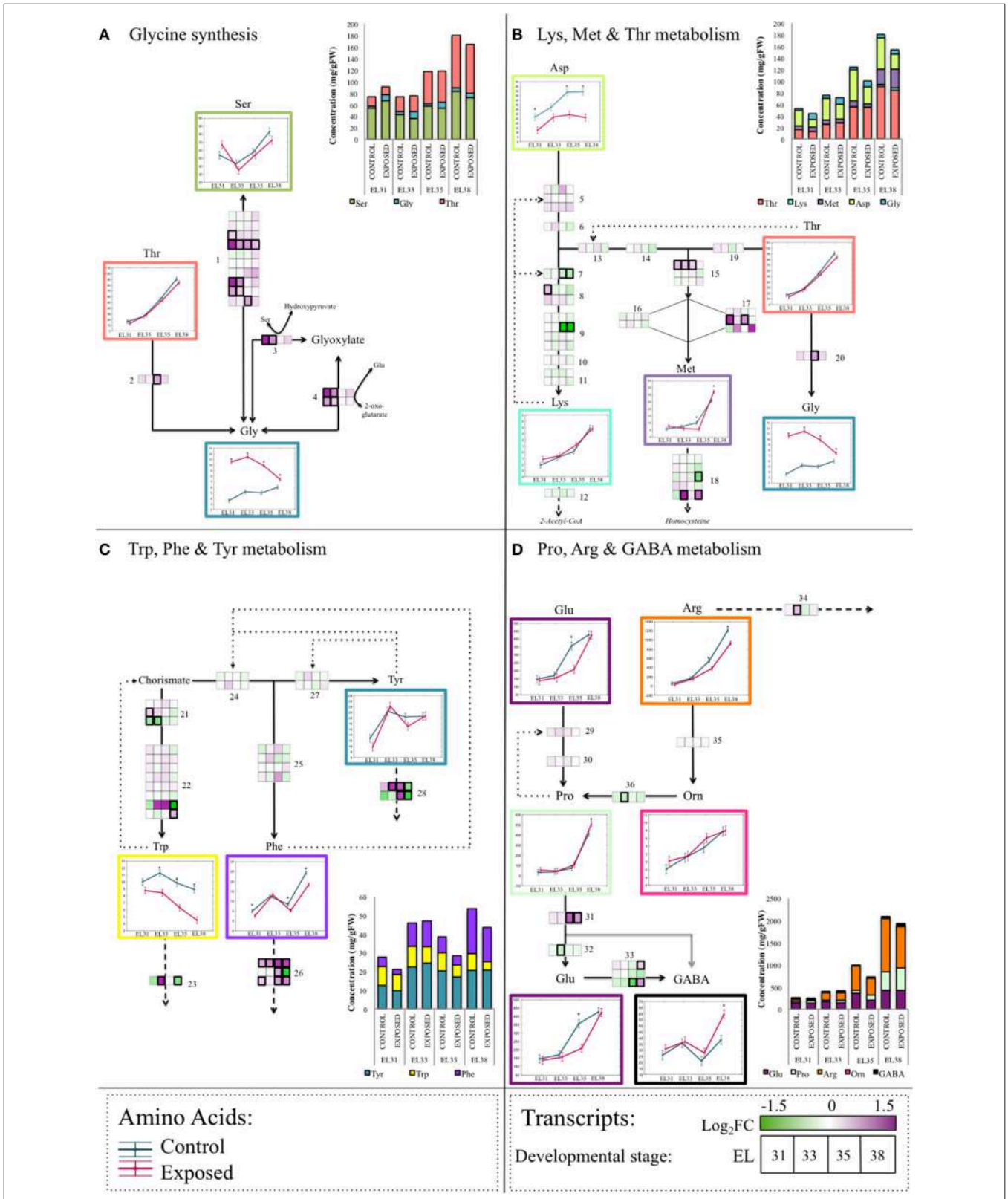


FIGURE 7 | A summarized schematic representation of the four amino acid metabolite and transcriptomic networks analyzed in this study. Enzymatic steps are indicated as black arrowed lines, spontaneous (non-enzymatic) metabolic processes are indicated by gray arrowed lines. **(A)** The network representing the various

(Continued)

FIGURE 7 | Continued
pathways involved in Gly synthesis. **(B)** The superpathway of Lys, Met, and Thr synthesis from Asp. **(C)** The superpathway of Trp, Phe and Tyr synthesis from chorismate. **(D)** The superpathway of Pro, Arg, and GABA metabolism. Dotted lines represent feedback inhibition loops, whereas striped lines represent catabolic pathways of amino acids not included in this diagram. Blocks indicate the mean-centered $\log_2$ fold change of the FPKM expression value of the specific transcript encoding the particular enzymatic step at each berry developmental stage when comparing exposed to control samples. Significant differences between FPKM expression values between exposed and control grapes at a particular developmental stage is indicated by a bold frame around the specific gene. Amino acid concentrations [mg/g fresh weight (FW)] are represented as ANOVA line-plots where significant differences ($q \leq 0.05$) between exposed and control grapes are indicated by an asterisks (*). Line graphs representing exposed and control samples are staggered along the x-axis representing the respective developmental stages. The genes represented by numbers are listed in **Table S9**.

VIT_13s0019g04540) and Phe (VIT_06s0004g02620, VIT_08s0040g01710, VIT_13s0019g04460) while upregulation of the same Phe catabolism genes facilitated the accumulation of higher levels of flavonols. The upregulation of a different set of Tyr catabolic enzyme genes (VIT_00s0394g00040, VIT_00s0225g00230, VIT_10s0116g01660, VIT_12s0028g00710, VIT_16s0039g01410) simultaneously contributed to the transcription of tocopherols that subsequently lead to the accumulation of elevated lipophilic antioxidant levels (L-ORAC) in green grapes exposed to elevated light (**Figure 8**).

DISCUSSION

Molecular profiling tools provide sensitive and comprehensive snapshots of how a plant/organ/tissue is responding at a specific point in time. It is quite obvious that the value of these molecular snapshots is amplified if they are framed by an accurate understanding of the environmental cues, the developmental stage and general plant status of the plant. This has lead to a renewed focus on integrating accurate measurements of environmental impact factors with grapevine phenotypes observed, specifically in grapevine berries. Several recent studies have advanced our understanding of berry development, ripening and reactions to stress signals and have convincingly shown that berries throughout their growth curve react to their microclimatic environments, but with different responses (Zenoni et al., 2010; Sweetman et al., 2012; Palumbo et al., 2014; Pilati et al., 2014; Wong et al., 2016). Interestingly, many of these studies also showed the resilience of berries to mitigate mild stresses (Carbonell-Bejerano et al., 2013; Martinez-Luscher et al., 2014; Rienth et al., 2014; Wu et al., 2014; Ghan et al., 2015; Suzuki et al., 2015; Joubert et al., 2016; Santo et al., 2016; Savoi et al., 2016; Young et al., 2016; Sun et al., 2017) leading to minimal impacts on overall berry growth and development. How this is orchestrated/managed was the focus of this study, and an experimental system that was previously proven to render grape berries more exposed to light, with minimal changes in berry temperatures, was used (validation of light as the main experimental parameter in the vineyard experiment was described in Young et al., 2016).

The Grape Berry Developmental Profile Remained the Strongest Transcriptional Driver Despite Elevated Light Exposure

Our data confirmed that development remained the strongest driver for the statistical separation of the grape samples based on their transcriptomes, regardless of viticulture treatment implemented. On average, not more than 8% of the berry transcriptome was affected by the elevated exposure at any of the developmental stages evaluated. As expected, berries in the green developmental stages were transcriptionally more similar in the global sense to each other than to berries from the ripening stages. Developmental phase-specific biomarkers were identified as genes that were responsible for the greatest transcriptional differences observed between green and ripening grape berries. Not only were 48 of the biomarkers identified in this study (**Figure S4**) also previously established as biomarkers by other research groups (Zamboni et al., 2010; Palumbo et al., 2014), but all, except nine of these genes, were unaffected by elevated light exposure at the stages when the berries were either green, ripening or throughout development.

Green Grapes Maintain Growth and Development by Protecting the Photosynthetic Machinery under Light Stress Conditions

It was previously shown that the exposed grape berries were not different from their control counterparts in terms of size and weight, sugar accumulation and acid degradation patterns (Young et al., 2016) and the transcriptional data also showed that gene expression associated with growth and development, and primary metabolism was not altered by the leaf removal treatment (this study). Despite this fact, photosynthesis-related gene expression, that forms part of primary metabolism, proved to be (the most) significantly affected by the treatment in green grapes.

Our data confirmed that the green berries responded to the increased exposure to try and mitigate the light stress—the first line of defense against potentially damaging effects of photodamage, was the simultaneous activation of several avoidance strategies. One of the strong reactions was the transcription and synthesis of phenolic compounds and tocopherols that were activated, presumably to maintain the redox balance.

Among the phenolic compounds that accumulated at higher levels in response to elevated light were hydroxycinnamic acids and flavonols. Both hydroxycinnamic acids and flavonols can limit photodamage through their ability to scavenge free radicals and ROS, thereby contributing to the maintenance of oxidative homeostasis (Tattini et al., 2005; Agati et al., 2007, 2012, 2013). Flavonols, however, additionally possess the ability to act as sunscreen molecules themselves. They

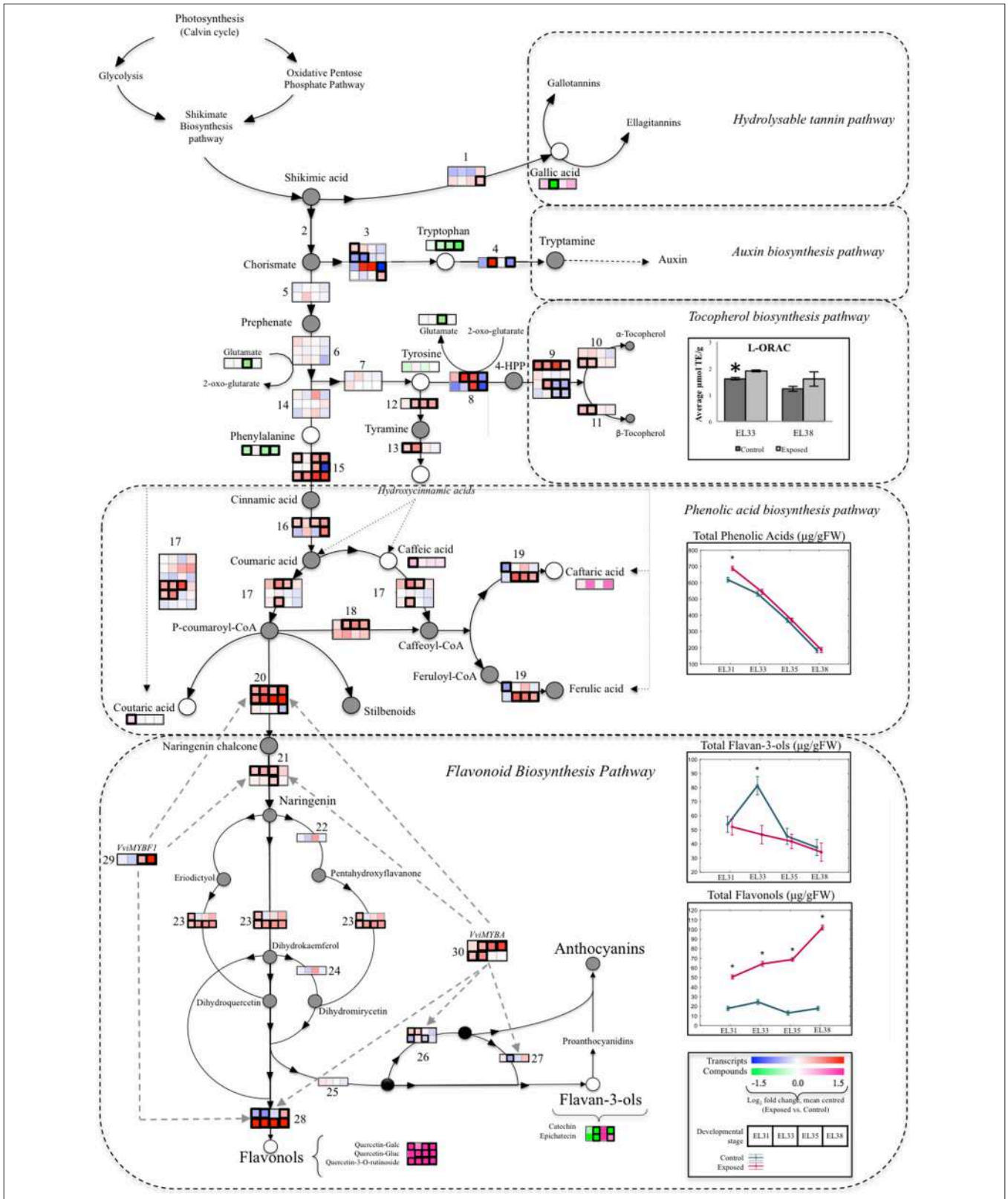


FIGURE 8 | A summarized overview of the branch points between primary berry metabolism toward the phenylpropanoid pathway overlaying transcriptomic and metabolomic data generated from exposed and control grapes harvested at each phenological stage. Blocks indicate the mean-centered $\log_2$ fold change of

(Continued)

FIGURE 8 | Continued
the FPKM expression value of the specific transcripts and metabolites involved in the particular enzymatic step at each berry developmental stage when comparing exposed to control samples. Significant differential expression ($q \leq 0.05$) of genes and compounds are indicated by a bold contour (frame). Total concentrations (μg/g FW) of phenolic acids, flavonols and flavan-3-ols are represented by ANOVA line-plots where significant differences ($q \leq 0.05$) between exposed and control samples are indicated by an asterisks (*). Line graphs representing exposed and control samples are staggered along the x-axis representing the respective developmental stages. Gray circles represented compounds that were not measured, whereas black circles represent various possible compounds at the same enzymatic step. Striped gray arrows represent regulatory steps by associated transcription factors. The genes represented by numbers are listed in **Table S10**.

achieve this by absorbing highly energetic solar wavelengths, thereby limiting the generation of ROS due to photooxidation. Although flavonol levels have been found to be negligibly low in developing grape berries, the transcription and subsequent accumulation of these compounds in both a light-dependent and development-independent manner have been reported and extensively characterized in grapes (reviewed by Downey et al., 2006; Czemmel et al., 2009; Matus et al., 2009; Malacarne et al., 2016; Yu et al., 2016; Pastore et al., 2017).

The other avoidance mechanism activated in the exposed berries was non-photochemical quenching, the process by which a large part of excitation energy generated by excessive light exposure can be dissipated as heat (via carotenoids). Through this process, the xanthophyll cycle is activated in which the xanthophyll pigment, violaxanthin, is de-epoxidized to zeaxanthin through the activity of the violaxanthin de-epoxidase (VDE) enzyme, thereby limiting energy transfer from LHCII to PSII. Although the carotenoid metabolites and their ratio's, as well as the transcriptional activation and elevated synthesis of the VDE enzyme already confirmed that the green berries have activated the xanthophyll cycle pigments (Young et al., 2016), the transcriptional mechanism of NPQ activation could be further explored in this study. The PsbS subunit of PSII has been established as the enzyme responsible for "sensing" the impending light stress and initiating NPQ (Li et al., 2000; Gregan and Jordan, 2016). The gene encoding the grapevine PsbS enzyme was found to be significantly upregulated by the leaf removal treatment from the onset of green berry development, potentially linking to the activation and upregulation of the VDE enzyme and subsequent increase of the xanthophyll pool as reported in Young et al. (2016).

However, as high levels of light exposure were maintained throughout the season, it appears that damage to the photosynthetic machinery could no longer be avoided through NPQ alone. At the second green developmental stage (EL33); the process of reversible photoinhibition (RPI) was subsequently activated in an attempt to no longer avoid, but rather acclimate to the continuous light stress, while the synthesis of other antioxidant molecules such as tocopherol and flavonols remained transcriptionally and metabolically upregulated (**Figure 8**, **Table S10**). RPI is the process in which photodamage is actively concentrated to the reaction-center binding D1 protein that forms part of Photosystem II (Kyle et al., 1984; Powles, 1984). In doing so, the rapid and ongoing turnover of the D1 protein is ensured through the disorganization of the PSII-LCHII supercomplex in order to remove and replace the damaged D1 protein with a newly synthesized copy. This results in the protection of the photosynthetic machinery from photooxidative stress.

These photoprotective strategies have been well characterized and extensively reported in vegetative tissues (leaves and stems) of numerous plant species (Li et al., 2000; Crouchman et al., 2006; Kato et al., 2012; Niyogi and Truong, 2013; Gorecka et al., 2014). To our knowledge, NPQ and RPI have not been thoroughly investigated in the context of green grape development.

Young et al. (2016) showed higher carotenoid levels (especially xanthophylls) in the exposed berries, yet chlorophyll a: chlorophyll b and total carotene: chlorophyll ratios were maintained in the earlier stages (up until véraison). Total chlorophyll, and the levels of the major photosynthetic carotenoids (β-carotene and lutein) were also not significantly affected. The authors concluded that a pool of carotenoids (predominantly xanthophylls) were responsive to the treatment and increased in response to the increased exposure (light). Since the major carotenoids and chlorophylls were seemingly unaffected, the authors concluded that the increased pool of xanthophylls were able to protect the photosynthetic machinery for normal development to proceed (without damage). The data presented here shows that on a transcriptional level the structural proteins of photosynthesis were significantly upregulated and indicated that there was a higher demand for these proteins possibly due to an increased turnover (damage and repair cycle). Kyzeridou et al. (2015) demonstrated the green fruits of *Nerium oleander* and *Rosa* sp. have a higher cyclic electron flow activity around PSI, when compared to leaves. Kotakis et al. (2006) further showed that cyclic electron flow is enhanced (at the expense of the linear photosynthetic electron flow) in twig collenchyma to adjust potential ATP/NADPH ratios and/or to counteract the detrimental effects of hypoxia. This, combined with the increased activity of non-photochemical quenching via the xanthophyll cycle observed in apple (Cheng and Ma, 2004) and grapevine (Young et al., 2016), suggest that non-foliar photosynthesis is possibly required to produce ATP in organs where gas exchange is prevented (Kalachanis and Manetas, 2010).

In this study, the sequential and simultaneous transcriptional activation of light stress mitigation mechanisms proved to be effective in avoiding irreversible photoinhibition and maintaining the development and growth of grapes. This was evident in the global transcriptional responses and the accumulation of AAs that remained predominantly unaffected by the treatment in the green berries. Furthermore, the AAs considered as oxidative stress markers, Pro and GABA, remained unaffected by the treatment in the green grapes despite elevated exposure to light.

This combination of NPQ, RPI and development-independent flavonol synthesis, although effective in stress mitigation and acclimation, appeared to be energetically costly to the developing green grapes. Firstly, hydroxycinnamic acids were no longer differentially accumulated in response to elevated light, although the transcription and accumulation of flavonols remained dramatically higher in exposed grapes (**Figure 8**, **Table S10**). This might be explained by the fact that these compounds compete for the same aromatic AAs, Phe and Tyr, as precursors. Results to this effect were previously reported in tomato leaves exposed to various abiotic stresses (Martinez et al., 2016). The authors demonstrated that flavonols were more effective in the maintenance of oxidative homeostasis than hydroxycinnamic acids when precursors were limited. Furthermore, the MYB transcription factors known to regulate the transcription of several enzymatic steps involved in flavonoid synthesis (Czemmel et al., 2009) were significantly upregulated at each of the developmental stages (**Figure 8**). Secondly, the upregulation of several AA catabolic enzymes were further testament to the limitations placed on grape berry energetic resources as a consequence of photoprotection. AAs are involved in highly regulated metabolic networks and are crucial for the synthesis of proteins whilst also acting as precursors for a myriad of downstream metabolic processes. AAs have not only been implicated in normal growth and development but also in stress tolerance as their degradation may provide energetic advantage to maintain stress response mechanisms which prove to be energetically expensive to plant metabolism under suboptimal growing conditions. In *Arabidopsis*, evidence exist that transcription of AA catabolic enzymes, with the exception of Pro catabolic enzymes, were more sensitive to abiotic stresses than that of the enzymes responsible for AA synthesis (Less and Galili, 2008). Caldana et al. (2011) showed that amino acid catabolism serves as the main cellular energy supply under adverse environmental conditions as inferred by high-density kinetic analysis. The activity of these catabolic enzymes could therefore provide metabolic energy generated from the breakdown of AAs for utilization toward maintaining plant primary metabolism under stressful biotic and abiotic conditions. Additionally, it has been proposed that excessive accumulation of the branched chain amino acids, or rapid protein turnover induced by adverse environmental conditions could potentially lead to cellular apoptosis as a result of respiratory oxidation. The catabolic breakdown of these AAs is seen as a necessary detoxification mechanism under these conditions, as observed in *Arabidopsis* cell cultures (Taylor, 2004). Since, the branched chain AAs did not accumulate differentially in our investigation (**Figure S5**) we, however, did not consider it the likely metabolic driver for the differential transcription of AA catabolic enzyme encoding genes.

Genes characterized in one of the aforementioned studies (Less and Galili, 2008) were utilized to identify homologous grapevine genes and their expression analysis in our investigation yielded similar results to previous reports. Transcription of the enzymes responsible for AA synthesis was predominantly unaltered by the elevated light exposure treatment whereas genes encoding the AA catabolic enzymes were far more sensitive to the treatment in comparison (**Figure 7**).

The catabolism of AAs during the green berry developmental stages therefore could have provided the green grapes with substrates necessary for downstream metabolic reactions when energetically costly abiotic stress protection mechanisms were simultaneously activated. These included the maintenance of nitrogen fixation that lead to slightly shifted substrate utilization and lower accumulation of Asn, Asp, and Gln levels. The accumulation of lower levels of Phe that serves as the precursor for flavonols necessarily synthesized to protect the grapes against elevated light, were also evident, similar to the mechanisms implemented by vegetative plant organs.

Significantly higher concentrations of Gly in response to the light treatment further substantiate the notion that green grapes respond to light stress as vegetative, source organs. Gly and the enzymes responsible for its decarboxylation, Gly decarboxylase complex (GDC) play an integral part in the successful functioning of photorespiration system. Increased photosynthesis and subsequent elevated levels of electron flow through the photosystems as a means to protect the photosynthetic machinery from light stress, is proposed to cause an altered redox state that ultimately influences the rate of photorespiration (Hutchison et al., 2000; Wingler et al., 2000; Voss et al., 2013). Despite elevated expression levels of the GDC encoding genes reported in our investigation (**Figure 7A**), the GDC themselves are prone to oxidation, hereby causing the accumulation of Gly under high light. Furthermore, Gly is considered to be the rate-determining compound in the synthesis of the antioxidant, glutathione, that might contribute to maintaining the oxidative homeostasis within the developing grape berry. This effect that elevated light exposure had on photorespiration and subsequent high Gly accumulation were previously reported in *Arabidopsis* (Caldana et al., 2011; Florian et al., 2014). To further support this proposed link between Gly and protection of the photosynthetic machinery in green grapes, the difference in the concentration of Gly when comparing exposed to control grapes become less significant as photosynthetic activity declines throughout berry development.

These findings established that green grapes responded to elevated light exposure by activating and refining stress mitigation strategies to predominantly protect the photosynthetic machinery similar to vegetative plant organs. In an attempt to prioritize growth and development, green grapes utilized and combined several precursor substrates and mechanisms to maintain photoprotection and the synthesis of flavonols, regardless of limited energetic resources.

Ripening Berries Do Not Effectively Mitigate the Effects of Light Stress

Véraison is the grape developmental stage during which the berry begins to transition from being a photosynthesizing, organ toward becoming a senescing organ while it retains metabolic characteristics of both berry developmental phases on a transcriptional level, as reported here. Véraison has further

been extensively characterized by an oxidative burst that includes the production of ROS (particularly H_2O_2) that serves as a signaling molecule to signify the initiation of the ripening (Pilati et al., 2007). It would be reasonable to expect that the production of low-levels of H_2O_2 as a consequence of light stress along with this developmentally driven oxidative burst could culminate toward a redox imbalance in berries exposed to elevated light. In contrast, the grapes that were exposed to elevated light at véraison did not accumulate higher levels of the known stress markers, Pro and GABA, however, at EL38, when the grapes were no longer photosynthetically active, these stress markers did accumulate at higher levels in exposed grapes. It would therefore be reasonable to speculate that this could be a reflection of the berries' successful limitation of the accumulation of ROS through the combination of NPQ, RPI and flavonol production until véraison (**Figures 4**, **6**, **8**).

The rapid accumulation of both Pro and the non-protein AA, GABA, have been extensively reported in plants exposed to abiotic stresses and the metabolism of these AAs are intimately linked (**Figure 7**). Pro has been shown to enhance primary photochemical activity of thylakoid membranes by limiting photoinhibition and its synthesis is highly sensitive to light (Alia et al., 1997). Furthermore, in grapevine leaves, it has been reported that Pro has the ability to limit inactivation of some antioxidant enzymes while further being capable of stimulating the expression of others (Agudelo-Romero et al., 2013). Therefore, the importance of Pro homeostasis, as opposed to its accumulation, in response to oxidative stress has gained particular interest in the context of plant abiotic stress response (Kavi Kishor and Sreenivasulu, 2014). The homeostasis of Pro levels was found to be imperative to actively dividing plant cells to sustain growth despite exposure to long-term stress. GABA, on the other hand, is capable of either contributing to plant abiotic stress response through its involvement as either a stress signal amplifier or in the maintenance of the carbon: nitrogen ratio under stressful conditions (Barbosa et al., 2010; Kinnersley and Turano, 2010). The accumulation of elevated levels of both Pro and GABA can therefore be associated with plants experiencing abiotic stress symptoms.

Similar to the earlier green developmental stages, the maintenance of photoprotective mechanisms throughout most of the berry development comes at an energetic cost to the grapes that are at this stage no longer accumulating precursors and energy at the rate that photosynthesizing organs are able to. This energetic strain on the grapes are reflected in lower levels of almost half of the AAs measured in these grapes as well as lower total AA concentrations overall measured in the grapes exposed to elevated light.

The transcription and accumulation of flavonols remained elevated in an attempt to protect the berries from light damage and at this stage, the antioxidant pool available to the ripe berries were additionally supplemented by higher levels of apocarotenoid accumulation as reported earlier (Young et al., 2016). Due to significantly higher transcription involved in photosynthesis-related proteins during the early developmental stages, combined with increased carotenoids provides a larger pool of substrates for the degradation via carotenoid cleavage enzymes (CCDs). This leads to an increased apocarotenoid pool in the later stages. Although these compounds are thought of as mere degradation products or volatile impact odorants; they also function as antioxidants and it is speculated that apocarotenoids may play an important signaling role in plant development and in responses to environmental stimuli (Avendaño-Vázquez et al., 2014; Hou et al., 2016).

Similarly, we hypothesize that higher concentrations of several AAs at EL38 (**Table 1**) in response to elevated light exposure may not be a consequence of transcription of the related biosynthetic enzyme genes at this late developmental stage, but rather due to the systematic degradation of higher protein levels synthesized during early development. The degradation of higher protein levels could therefore liberate higher levels of the respective AA constituents. The dramatic and consistent upregulation of numerous heat shock proteins throughout berry development (**Table S7**) further supports this hypothesis because of their well-established role as molecular chaperones associated with protein recycling in response to abiotic stress in other plant models as reviewed in Wang et al. (2004).

This systematic shut-down of the protection strategies as the grapes reach maturity were further evident by the fact that the lipophilic antioxidant capacity (L-ORAC) of these grapes were no longer elevated significantly and that Pro and GABA levels were significantly higher in exposed compared to control grapes at this stage. Although the oxidative homeostasis of these grapes were no longer entirely intact (as evident by elevated Pro and GABA levels), it is however important to consider that despite the light-induced stress status of these grapes at EL38, the sole purpose of the fruit had been achieved in the successful development and maturation of the grape seed. The redox-balance and stress responses of the grape berry were no longer of critical importance to the final development of the fruit as evident by the fact that the exposed and control grapes were not physically distinguishable when they were ripe.

CONCLUSION

In this study, we aimed to determine how developing Sauvignon blanc grapes manage to maintain primary metabolism and development despite being exposed and responding to non-lethal light stress. Our approach was to explore the global transcriptional response of grapes sampled from a highly characterized vineyard to determine how these grapes acclimated to light stress on a transcriptional level and to elucidate the metabolic consequences of these transcriptional changes. This approach allowed us to demonstrate that a leaf removal treatment in the berry bunch zone of developing Sauvignon blanc grape berries lead to the activation and refinement of several stress avoidance and tolerance strategies in parallel for the purpose of mitigating the effects of light stress whilst maintaining the normal developmental program of the grapes.

These results revealed that photosynthetically active berries are successful at mitigating the effects of light stress much like other vegetative plant organs by potentially limiting the synthesis and distribution of potentially harmful ROS through the continuous turnover of the photosynthetic machinery and

the production of light-absorbing flavonoid compounds as well as higher levels of carotenoids in green berries and subsequent apocarotenoids in ripe berries. These grapes achieved a state of acclimation through the redistribution of energy resources in the form of AA catabolism that provided energy precursors and substrates that contributed to the maintenance of these energetically costly stress mitigation mechanisms. To this end, green, photosynthesizing grapes maintain growth and development at all costs to protect the development and maturation of the grape seed.

AUTHOR CONTRIBUTIONS

MV and PY conceptualized and planned the study. PY implemented and maintained the viticultural treatments and was responsible for the berry sampling. KdP performed RNA processing and RNASeq data analysis. HE performed HPLC analysis for AAs and phenolic compounds. KdP, PY, and MV drafted the original manuscript and all authors contributed and finalized the publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01261/full#supplementary-material

Figure S1 | Pearson correlation matrix representing the entire transcriptomes of the initial 24 samples representing 3 biological replicates from control and exposed grapes at four phenological stages.

Figure S2 | ReviGO analysis output of GO enrichment data generated from the 5050 genes in the grapevine genome that was not expressed whatsoever in the grapes investigated in this study.

Figure S3 | Summarized results generated from Real-time PCR analysis.

Figure S4 | Venn diagram comparing the molecular biomarkers generated in this study to previously published biomarkers from Zamboni et al. (2010) and Palumbo et al. (2014) and differential expression analysis of biomarkers shared between this investigation and previously published biomarkers.

Figure S5 | The amino acid super pathway of Ile, Val, and Leu biosynthesis.

Table S1 | Primers used for Real-time PCR.

Table S2 | A table summarizing the retention times of phenolic compounds measured.

Table S3 | Summary of RNASeq reads and mapping.

Table S4 | List of positive and negative molecular biomarkers separating green (EL31 and EL33) from ripening (EL35 and EL38) berries.

Table S5 | Table listing the genes most significantly up and downregulated at each developmental stage ($-2 > Log_2FC > 2$).

Table S6 | Table listing all significantly differentially expressed genes ($q \leq 0.05$; $1.5 \leq Log_2FC \leq -1.5$) significantly correlated to predetermined gene expression clusters according to STEM analysis.

Table S7 | Functional annotation (Grimplet et al., 2012) of each of the genes that were highly upregulated ($2 \leq Log_2FC \leq -2$) between two or more phenological stages indicated in color as represented in **Figure 5**. *Q*-values represent the level of significant difference between the expression of each indicated gene at the specific developmental stage. Asterisks (∗) indicate multiple genes represented by the same functional annotation with *Q*-values in this case indicative of the average value of the multiple genes sharing the same function.

Table S8 | The amino acid concentrations of all the exposed and control grapes sampled from EL31, EL33, EL35, and EL38. D.N.Q. refers to AA concentrations that were detected but were at concentrations below the limit of quantification.

Table S9 | Genes involved in amino acid synthesis and catabolism as indicated by the numbers assigned in **Figure 7**.

Table S10 | The metabolite concentrations and gene expression levels as indicated by the numbers assigned in **Figure 8**.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Addendum A to Chapter 3

This Addendum contains relevant and additional data not shown in Chapter 3.

A highly characterised model vineyard approach towards effective implementation of "field-omics" in grapevine studies

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Introduction

It is proposed that one of the greatest challenges facing the grape industry during this century will be maintaining high quality sustainable grape production in a changing environment. Grape quality is a complex and somewhat elusive term associated with the composition of the berries and can be considered as a function of the multiple confounding variables that influence berry development in the heterogeneous vineyard environment[1]. These variables include the macro-, meso- and microclimate of the vines, amongst a myriad of factors that complicate the integration and utilisation of large datasets generated from field studies[2].

Aim:

- To utilise the concept of a model vineyard through extensive characterisation of geospatial patterns, plot layout and climatic factors that may influence the variability of berry quality

The Model Vineyard Concept

Geospatial patterns & Plot layout

Row 4	P8	P7	P6	P5	P4	P3	P2	P1	P0
Row 5	P8	P7	P6	P5	P4	P3	P2	P1	P0
Row 6	P8	P7	P6	P5	P4	P3	P2	P1	P0
Row 7	P8	P7	P6	P5	P4	P3	P2	P1	P0
Row 8	P8	P7	P6	P5	P4	P3	P2	P1	P0
Row 9	P8	P7	P6	P5	P4	P3	P2	P1	P0

SHADED: no leaf removal
EXPOSED: leaf removal

The checkerboard plot layout accounts for inter- and intravine variability by positioning treatment and control vine panels directly adjacent to each other hereby allowing each treatment panel to be bordered on each side by control panels and vice versa.

Predefined Viticultural Practices

Treatment: Partial leaf removal in the bunch zone

Characterisation of the environmental factors include evaluation of:

- Relative humidity
- Disease status
- Temperature (meso-, micro- & macroclimate)
- Wind
- Light intensity
- Stem water potential

Sampling strategy and berry characterisation

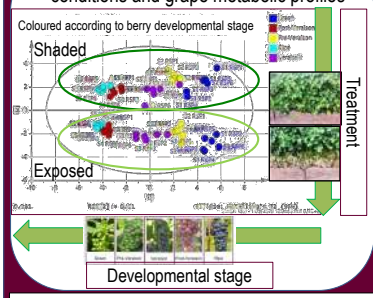
Berries are sampled at five developmental stages:

Metabolic profiling of the berries include analysis of:

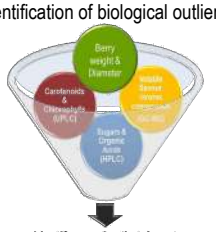
- Berry weight and diameter
- Carotenoids and Chlorophylls – UPLC
- Sugars and Organic acids – HPLC
- Volatile Flavour and Aroma compounds – GC-MS

Implementation of metabolic profiling data

Correlation between microclimatic conditions and grape metabolic profiles



Identification of biological outliers

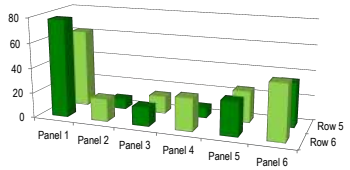


Identify samples that do not "behave"

Calculating the frequency at which each panel deviates one standard deviation point from the mean throughout metabolite profiling data to identify biologically relevant subsamples for downstream transcriptomic analysis.

Identification of sub-samples for down-stream analyses

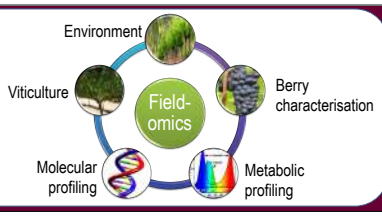
The percentage of instances that each panel behaves as a biological outlier:



Row 5	62	8	14	8	25	35
Row 6	78	18	15	26	27	44

Conclusions

Through the implementation of the model vineyard approach correlations could be made between the microclimatic conditions of the vines and the metabolic profiles of the resulting grapes. These findings contributed to the elucidation of statistically relevant subsamples within the vineyard layout that show limited biological variability for utilisation in further, highly sensitive molecular profiling analyses. By integrating these profiles generated from grape berries harvested from this model vineyard, patterns associated with specifically implemented viticultural practices could be distinguished from predetermined developmental patterns. This study contributes to our efforts to establish effective workflows for omics technologies in complex vineyard environments.



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Addendum B to Chapter 3

This Addendum contains relevant and additional data not shown in Chapter 3.

Grapevine Plasticity in Response to an Altered Microclimate: Sauvignon Blanc Modulates Specific Metabolites in Response to Increased Berry Exposure[1]

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In this study, the metabolic and physiological impacts of an altered microclimate on quality-associated primary and secondary metabolites in grape (*Vitis vinifera*) 'Sauvignon Blanc' berries was determined in a high-altitude vineyard. The leaf and lateral shoot removal in the bunch zones altered the microclimate by increasing the exposure of the berries. The physical parameters (berry diameter and weight), primary metabolites (sugars and organic acids), as well as bunch temperature and leaf water potential were predominantly not affected by the treatment. The increased exposure led to higher levels of specific carotenoids and volatile terpenoids in the exposed berries, with earlier berry stages reacting distinctly from the later developmental stages. Plastic/nonplastic metabolite responses could be further classified to identify metabolites that were developmentally controlled and/or responded to the treatment in a predictable fashion (assessed over two consecutive vintages). The study demonstrates that grapevine berries exhibit a degree of plasticity within their secondary metabolites and respond physiologically to the increased exposure by increasing metabolites with potential antioxidant activity. Taken together, the data provide evidence that the underlying physiological responses relate to the maintenance of stress pathways by modulating antioxidant molecules in the berries.

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M.A.V. and A.D. conceptualized the initial study; P.R.Y., E.A., D.A.J., and M.A.V. were involved in the experimental layout; Z.C., P.R.Y., and A.D. implemented and monitored the vineyard and viticultural treatments and maintenance; P.R.Y., E.A., Z.C., and D.A.J. did the field sampling; Z.C. did the climatic calculations and stem water potential measurements; P.R.Y. and Z.C. processed and analyzed the temperature and light data; E.A. and P.R.Y. processed the grape samples; E.A. characterized the berries; P.R.Y. performed the pigment extractions; H.A.E.-B. performed the ultra-high-performance liquid chromatography analysis; P.R.Y. created the pathway visualization; D.A.J. provided statistical and computational support for the study; P.R.Y. and H.A.E.-B. processed the samples for sugar and organic acid extractions and performed the HPLC analysis; K.d.P. performed the RNA extraction and transcriptome analyses; P.R.Y. and M.A.V. drafted the initial article; all authors contributed to discussion of the results, reviewing of the article, and approved the final article.

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Vineyards are highly variable environments where the plant must respond to changes within and across seasons. Grapevine (*Vitis vinifera*) berry ripening occurs over months, and the final berry composition is the expression of the interaction between the specific genotype (cultivar) and the environment over time (vintage). The grape and wine industries rely on cultivars and clones that have been purposefully selected and domesticated for thousands of years based on predominantly observable phenotypes (color, flavor/aroma, and/or survival [i.e. resistance to biotic/abiotic stresses]; Terral et al., 2010; Bouby et al., 2013). The genetic basis of these traits obviously underpins a biological function in the plant, but these functions and underlying mechanisms are still relatively poorly studied in grapevine.

The mechanism of phenotypic plasticity, defined as the capacity of a genotype to modulate its phenotypes under variable environmental conditions, is of specific interest in plant physiology. The observed phenotypic variations are due to differential regulation of the expression and/or function of genes involved in so-called plastic traits by the environment (Schlichting, 1986; Schlichting and Smith, 2002; Via and Lande, 2013). Transcriptomic plasticity has been demonstrated previously in grapevine 'Corvina', and candidate genes potentially involved in phenotypic plasticity have been putatively identified (Dal Santo et al., 2013). Those authors demonstrated that specific candidate plastic transcripts were associated with groups of vineyards

(i.e. a single genotype, cv Corvina) sharing common viticulture practices and/or environmental conditions, and plastic transcriptome reprogramming was more intense in the years characterized by extreme weather conditions. In a follow-up study, the variability in the observed metabolic plasticity of cv Corvina berries was illustrated in a comprehensive multiple vintage study (Anesi et al., 2015). Berry metabolites displaying terroir-specific signatures (and not year-to-year/vintage variation) were identified. The metabolites characterizing each of the macrozones included specific stilbenes, flavonoids, and anthocyanins (Anesi et al., 2015).

These studies and results further suggest that human intervention (e.g. via viticultural manipulations) combined with the prevailing environmental condition indelibly affects berry composition through changes in transcription that subsequently affect enzyme activity and/or the kinetics of biochemical reactions in the developing berry. Berry composition is not static and can be differentially modulated, thereby providing scope for human intervention in influencing and directing berry metabolism. Linking specific treatments conclusively to physiological mechanisms and metabolic impacts is required to address the questions of what, how, and, most importantly, why these changes occur to identify their underlying biological relevance.

In viticulture, one of the commonly used industrial practices involves canopy manipulations, such as leaf removal. It is not unique to grapevine and is used in many cultivated fruit crops for a variety of reasons that include (1) balancing vegetative growth and fruit production (crop load; Gordon and Dejong, 2007), (2) facilitating fruit collection (via training/trellising), (3) maximizing light incidence (via trellising/training and/or leaf removal; Stephan et al., 2008), and (4) pest control (by improving air flow and light penetration in the canopy; O'Neill et al., 2009).

Leaf removal has been used for diverse purposes, usually with a predisposed viticultural and/or oenological outcome, for example: (1) crop reduction (via early prebloom leaf removal) in high-yield cultivars (Reynolds and Wardle, 1989; Palliotti et al., 2012); (2) improving the quality of grapes (where quality is defined as acid balance and lower pH juice [via a predominantly higher tartrate content]; Hunter and Visser, 1990; De Toda et al., 2013); (3) decreasing fungal infection (usually *Botrytis* spp.) by improving air flow (in this context, healthy grapes are associated with quality; English et al., 1989; Gubler et al., 1991; Staff et al., 1997); (4) improving the sensory perception of the resultant wines (typically described as a reduction in the perception of the green character in both white [e.g. cv Sauvignon Blanc] and red [e.g. cv Cabernet Sauvignon] wines, or as an increase in tropical attributes [typically in white cultivars, such as cv Sauvignon Blanc]; Staff et al., 1997; Tardaguila et al., 2008; Šuklje et al., 2014); and (5) improving the color stability of wines from red cultivars (Chorti et al., 2010; Sternad Lemut et al., 2011; Lee and Skinkis, 2013). Typically, however, these studies report a vintage effect (i.e. an inconsistent/irreproducible effect and/or unclear results, referred to as slightly significant effects and/or tendencies, between consecutive years of experimentation), or conflicting data are obtained from different cultivars or the same cultivar in different geographical locations (for review, see Kuhn et al., 2014).

Although this specific viticultural treatment is widely used in viticulture, it has not yet conclusively been linked to a physiological mechanism(s) and metabolic impacts in grapevine berries. Our aim with this study was to apply a field-omics workflow (seeking a causal relationship between a viticultural treatment, the microclimate, and metabolic responses at different stages of berry development) to characterize the physiological outcome(s)/mechanisms of a targeted leaf removal in the bunch zone. The principles and benefits of this type of approach are outlined by Alexandersson et al. (2014). The impact of the leaf removal treatment, performed at an early phenological stage, was characterized by quantifying the abiotic (environmental) variables in the bunch zone (i.e. microclimate) in a characterized commercial experimental vineyard. The consequent impact on berry composition was measured by focusing on the primary and secondary metabolites typically associated with quality parameters, namely (1) sugars and organic acids, (2) carotenoids, and (3) volatile terpenoid-derived flavor and aroma compounds (predominantly monoterpenes and norisoprenoids). The results showed that pools of specific metabolites were under comparatively strict developmental control (e.g. sugars, organic acids, chlorophylls, and the major carotenoids), whereas other metabolites (e.g. specific xanthophylls, monoterpenes, and norisoprenoids) responded to the altered microclimate (i.e. increased exposure) differentially and displayed developmental stage-specific phenotypic plasticity. Pathway analysis of the genes and metabolites involved in the carotenoid metabolic pathway was subsequently performed to verify the observed metabolic response(s). This study led to a proposal that the impact of the treatment can be explained by a mechanism of antioxidant homeostasis maintenance in the berries experiencing increased exposure.

RESULTS

Quantitative Characterization of the Macroclimate in the Model Vineyard

An overview of the research methodology is outlined in Supplemental Figure S1. The Elgin region and vineyard site were classified according to viticultural climatic indices based on weather station data (i.e. regional macroclimatic) and mesoclimatic data (i.e. local vineyard). The indices selected for characterization are typically used to categorize the climatic potential of a region or vineyard (for grape growing) and, therefore, are indirectly linked to the characteristics and qualitative potential of grapes (Tonietto and Carbonneau, 2004). The various classification indices characterize the Elgin region as a temperate region with moderate to cool

nights (Supplemental Table S1). At more than 250 m above sea level, Elgin is a high-altitude wine-grape-growing region in South Africa. This elevation and the proximity to the cold Atlantic Ocean (and subsequent exposure to the cooling sea breeze) make it the fourth coolest wine-grape-growing region in South Africa. This site was chosen as a typical moderate climatic site for the production of a commercially desirable style of cv Sauvignon Blanc wine. The altitude and moderate climate minimized the potential for sunburn damage of berries in the leaf removal-treated vines.

Quantitative Characterization of the Microclimate in the Bunch and Canopy Zones Confirmed Increased Exposure for the Treated Berries

Leaf removal is typically used in viticulture to increase the photosynthetic active radiation (PAR) reaching the bunch zone and/or to decrease humidity at the fruit level. The light exposure in the bunch zone was strongly modified by the leaf removal treatment, with average light intensity (PAR) values of 52% ± 14% (average percentage PAR relative to the ambient, full sunlight [100%] at the date and time of sampling) for all cloudless sampling dates (Fig. 1). Conversely, the control bunches intercepted significantly less incoming radiation (PAR values of 4% ± 2%, relative to 100% ambient, full sunlight). Bunches in the exposed panels, therefore, received significantly more (seasonal average of more than 10 times higher) light than the shaded control bunches.

The daily average temperatures in the bunch zones of the respective treatments for the growth period (season) were not statistically significantly different when the data were considered on a daily mean hourly basis across the complete season (Fig. 2A). The temperature differences within the bunches of the treatments were insignificant throughout the entire season, ranging from a daily minimum of 11.4°C to a daily maximum of 38.2°C with a mean of 21.5°C ± 5.3°C for the exposed bunches, versus a range of 11.5°C to 37.7°C with a mean of 21.5°C ± 5.2°C for the bunches in the control treatments. Interestingly, the temperature in the canopy (above the bunch zone) of the exposed treatments was higher than that from the canopy of the control vines (Fig. 2B). Significant differences, however, could only be seen in the nighttime canopy temperatures (i.e. from sunset to sunrise), with the exposed canopies displaying higher temperatures than the control canopies, possibly indicating increased reflectance from the soil. This result was also shown in the seasonal thermal unit accumulation for the canopy and bunch temperatures, with significant seasonal differences only in the canopy temperatures (Fig. 2C). No significant differences in daytime canopy temperatures or bunch temperatures (per treatment) were found (Fig. 2).

It is understandably difficult to separate the effects of light from temperature in field experiments, since exposure to sunlight invariably results in increased temperatures. ANOVA and statistical testing were used to evaluate light and temperature as environmental factors potentially altered by the treatment. Supplemental Figure S2 shows the contribution of canopy temperature, bunch temperature, and light to the observed variance.

Leaf Removal Did Not Affect the Berry Physical Characteristics or the Ripening Dynamic of the Berries

Berry weight and diameter were measured for all the berries sampled for metabolite analyses. The relationship between berry weight and diameter showed a positive linear relationship (r^2 = 0.99) across all developmental stages, irrespective of the treatment. There were no significant differences between the control and exposed berries (Supplemental Fig. S3). Major sugars (Glc and Fru) and organic acids (tartaric acid, malic acid, and succinic acid) concentrations in berries were measured at five developmental stages (Supplemental Fig. S4, A–E). In berries, the changes in major sugars and organic acids are well described, with the sugar concentrations accumulating as ripening progresses and the total organic acid concentrations decreasing. Glc was the most abundant hexose in the earlier stages of development (Eichhorn-Lorenz [EL] stages EL31 and EL33), but from véraison (EL35) until harvest (EL38), Glc and Fru were present in approximately equal ratios.

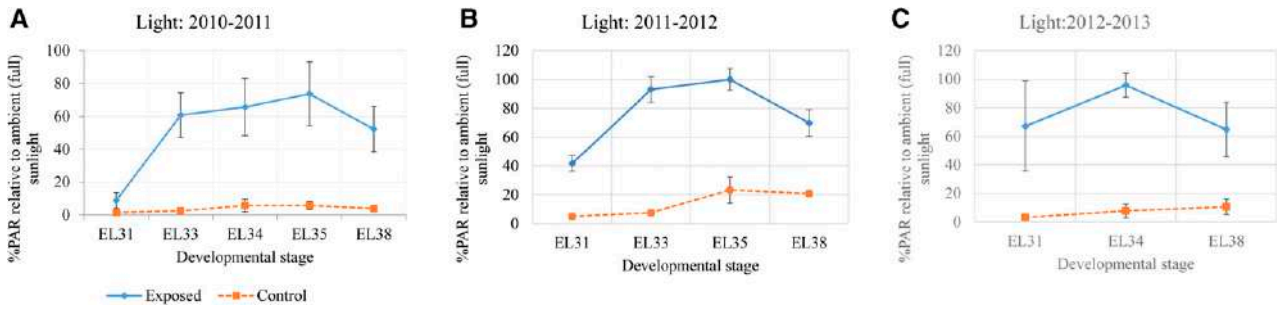


Figure 1. Characterization of the microclimate: light. PAR is shown in the bunch zone at the time of sampling for the respective sampling days for 3 consecutive years (vintages): A, 2010-2011; B, 2011-2012; and C, 2012-2013. Only cloudless days are represented.

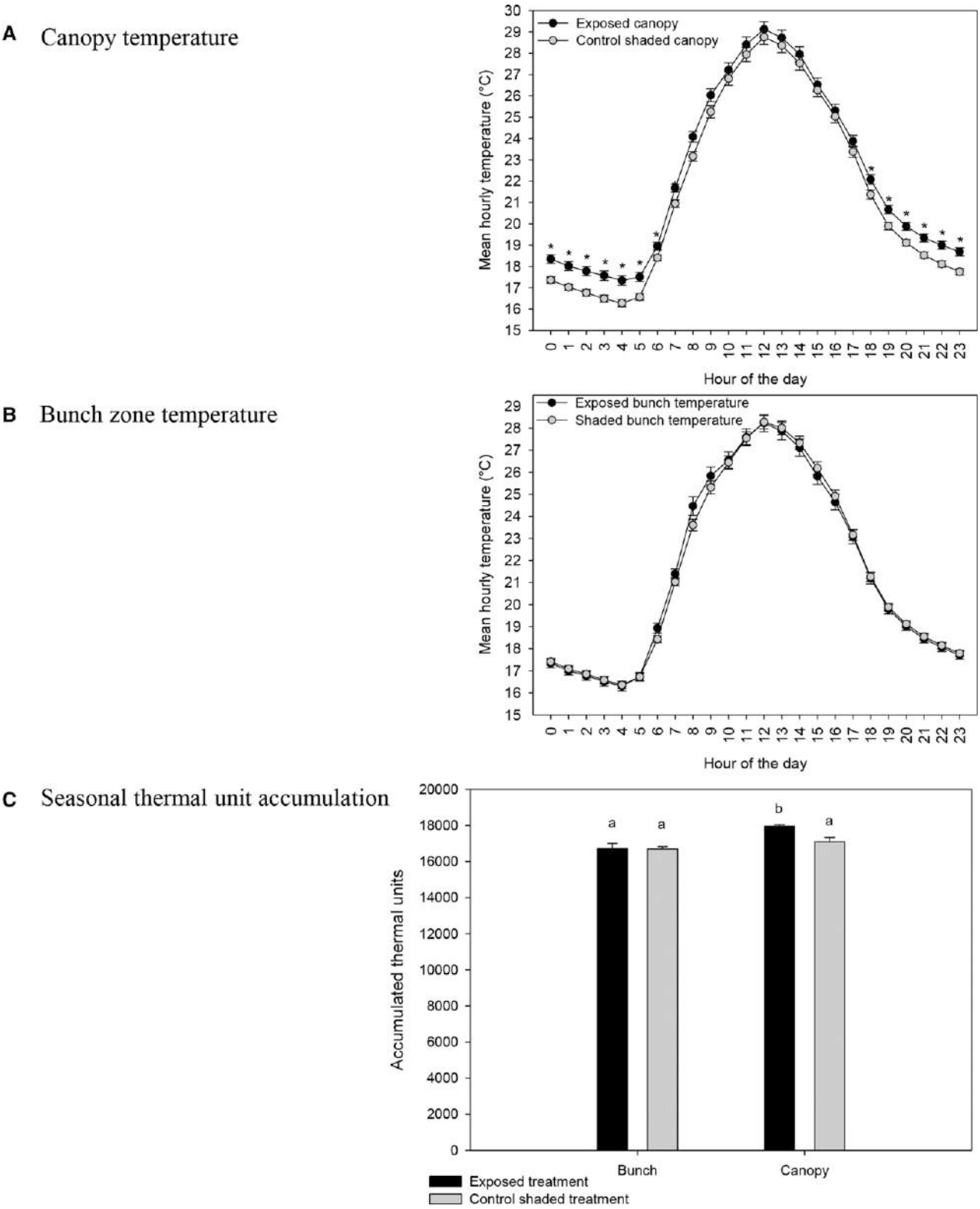


Figure 2. A and B, Hourly average temperature data in the canopy (A) and the bunch zone (B) in the exposed and control vines. Hours that are statistically different ($P \leq 0.05$) are indicated with asterisks. C, Mean thermal unit accumulation for the canopy and bunches. Different letters indicate significant differences (calculated with Fisher's LSD) between treatments, where $P = 0.05$.

The individual sugars and organic acids were not significantly affected by the leaf removal treatment in all but the EL38 developmental stage, in which a slight difference was shown (Supplemental Fig. S4E).

Developmental and Treatment-Specific Patterns of Metabolites Were Evident

Principal component analysis (PCA) and hierarchical clustering analysis were two of the data-mining tools used to reduce the complexity of the metabolite data. Metabolite analysis of field samples is typically hampered by inherent biological variation. Each panel analyzed in this study represents a unique biological entity, standard data interpretation potentially results in the loss of biologically relevant data (e.g. due to averaging), and potential correlations to the measured environmental variables can be blurred. Multivariate data analysis (e.g. PCA) reduces data complexity and can be used to identify the variables (metabolites in this study) that contribute the most to the optimal model. Unsupervised PCA plots were used to visualize the metabolite data (Supplemental Fig. S5), and separation was observed for developmental stages (EL31–EL38; PC1 on the horizontal axis) as well as treatment (exposed versus control samples; PC2 on the vertical axis). The increase in Glc and Fru, and inversely the decrease in chlorophylls (chlorophyll *a* and *b*) and the majority of the photosynthetic carotenoids (i.e. β-carotene, lutein, and neoxanthin), during ripening drove the developmental stage separation (considering PC1). The compositional differences in specific carotenoids (most notably the xanthophylls zeaxanthin, antheraxanthin, and lutein epoxide) and specific monoterpenes were predominantly responsible for the treatment separation on PC2 (Supplemental Fig. S5).

PCA is particularly useful for simplifying and visualizing data sets and helps to identify potential correlations in the underlying data sets. The associated scores and loadings plots are then used to identify correlations. The loadings plot relates to the variables and is used to explain the positions of observations in the scores plot. The scores plot relates to the observations, separates signal from noise, and is used to observe patterns and clustering in the observations. Whereas PCA models are unsupervised and find the maximal variation in the data, orthogonal partial least squares (OPLS) models are supervised prediction and regression methods. Orthogonal partial least squares-discriminant analysis (OPLS-DA) is used to analyze the relationship between the quantitative data matrix, x (i.e. the measured variables [e.g. metabolite concentration and/or transcript levels]), and a vector, y, containing qualitative values (i.e. the data descriptors or classes [e.g. developmental stages {EL31–EL38} or treatment {control or exposed}]). Separate OPLS models were generated to analyze the developmental and treatment class separations to identify the variables statistically contributing to the optimal models for (1) developmental stage discrimination (Fig. 3) and (2) treatment discrimination (Fig. 4).

The metabolites contributing the most to the model for developmental discrimination (Fig. 3) were the organic acids malic acid and succinic acid (and the associated total organic acid pool) and the monoterpenes trans-linalool oxide and eucalyptol (and the associated total monoterpene pool). The metabolites contributing the most to the model for treatment (exposure; Fig. 4) discrimination were the xanthophylls zeaxanthin and antheraxanthin (and the associated De-epoxidation state ratio and total xanthophyll pool) and the norisoprenoids geranylacetone and 6-methyl-6-hepten-2-one (MHO; and the associated total norisoprenoid pool).

Hierarchical cluster analysis was subsequently used to identify profiles (clusters) with similar trends between the analyzed metabolites (Fig. 5). A number of clusters were of particular interest: (1) metabolites showing a predominant developmental trend (Fig. 5, clusters 2, 4, and 6); (2) metabolites showing a predominant treatment effect (Fig. 5, clusters 1, 3, and 7); and (3) metabolites showing both developmental and treatment effects (Fig. 5, clusters 1, 2, 3, 5, and 6). The responses of the measured metabolites typically varied between the different developmental stages, with the early stages (EL31 and EL33) and the later stages (EL35 and EL38) generally responding similarly and with véraison as a transition stage (between the early/green and late/ripe stages).

Metabolites showing the developmental trend (Fig. 5, clusters 1 and 2) could be further subgrouped into metabolites that increased with development progression (Fig. 5, cluster 6) and metabolites that decreased with development progression (Fig. 5, clusters 2 and 4). The major sugars (Glc and Fru), MHO, and three monoterpenes (geraniol, linalool, and nerol) increased with developmental stage (similar to berry weight and diameter in the same cluster). It is important to note that hierarchical cluster analysis relies on Pearson correlation coefficients to match trends and does not discriminate similar trends that differ in amplitude. This is evident in the line graphs of geraniol, linalool, and nerol (Fig. 6), where both the control and exposed display upward developmental trends but the absolute values of the respective metabolites in the exposed berries were significantly higher (than the control). Chlorophylls *a* and *b* and the major carotenoids (e.g. lutein and β-carotene, representing approximately 80% of the total carotenoids), however, decreased concomitantly throughout development (Fig. 6A). The major organic acids (i.e. malic acid, succinic acid, and tartaric acid), as well as the xanthophyll neoxanthin and the norisoprenoid (apocarotenoid) β-ionone, displayed a similar developmental decrease (Fig. 5, clusters 2 and 4).

A cluster of three carotenoid-derived apocarotenoids (i.e. norisoprenoids; pseudo-ionone, β-damascenone, and geranylacetone) were characterized by an early-stage (EL31 and EL33) developmental pattern followed by a treatment-related response (from EL34/véraison),

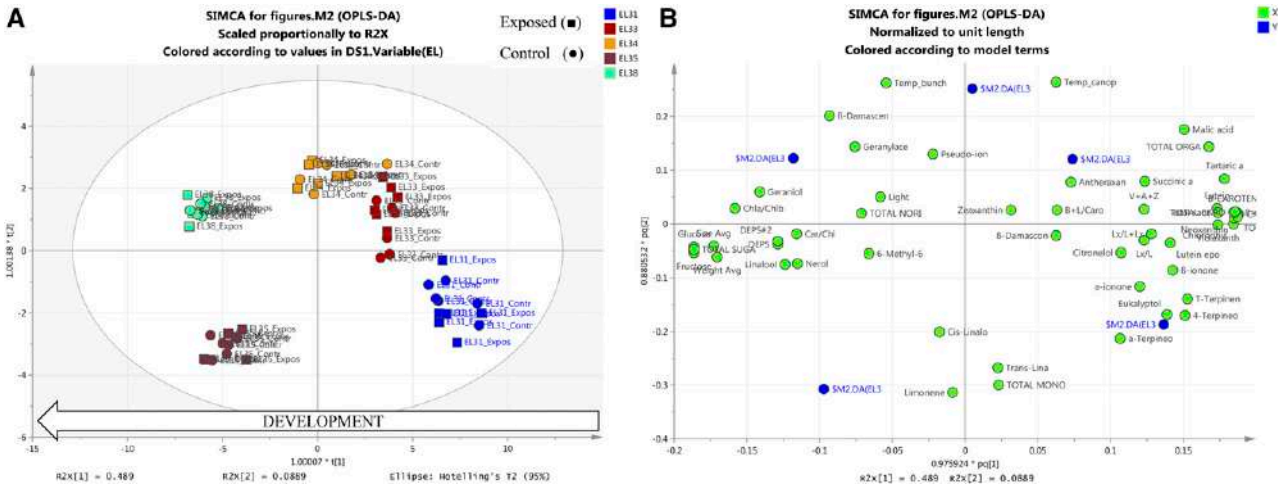


Figure 3. Supervised (developmental stage) OPLS of all metabolites from all stages. A, Scores plot for the respective samples. Samples are colored by developmental stage; control samples are indicated by circles and exposed samples by squares. B, Loadings plot for the measured variables in green and discriminant classes/categories in blue.

with higher levels in the samples from the exposed versus control bunches and positively correlated to the bunch temperature (Fig. 5, cluster 5).

The monoterpenes α-terpineol and trans-linalool oxide displayed a biphasic treatment effect, with higher levels in both the exposed berries (versus the control berries) in the early (EL31) and late (EL35 and/or EL38) stages, with insignificant differences in the midripening stages (EL34 and/or EL35; Fig. 5, cluster 8). The xanthophylls antheraxanthin and zeaxanthin showed a clear treatment effect, with higher levels in the exposed berries (versus the control) in all developmental stages (EL31–EL38). The treatment effect was significantly greater in the early stages (EL31 and EL33) versus the later stages (EL34, EL35, and EL38; Fig. 5, cluster 7).

Sugars and Organic Acids Are Predominantly Developmentally Regulated

It is interesting that the Glc and Fru concentrations in the berries were present in equal proportions (Glc:Fru ratio approximately 1) only from véraison (EL35) onward (Supplemental Fig. S4D). In the earlier stages, however, Glc is the dominant hexose. In the EL31 stage, no Fru could be detected. A Glc:Fru ratio of approximately 1 illustrates that Glc and Fru in the berries are

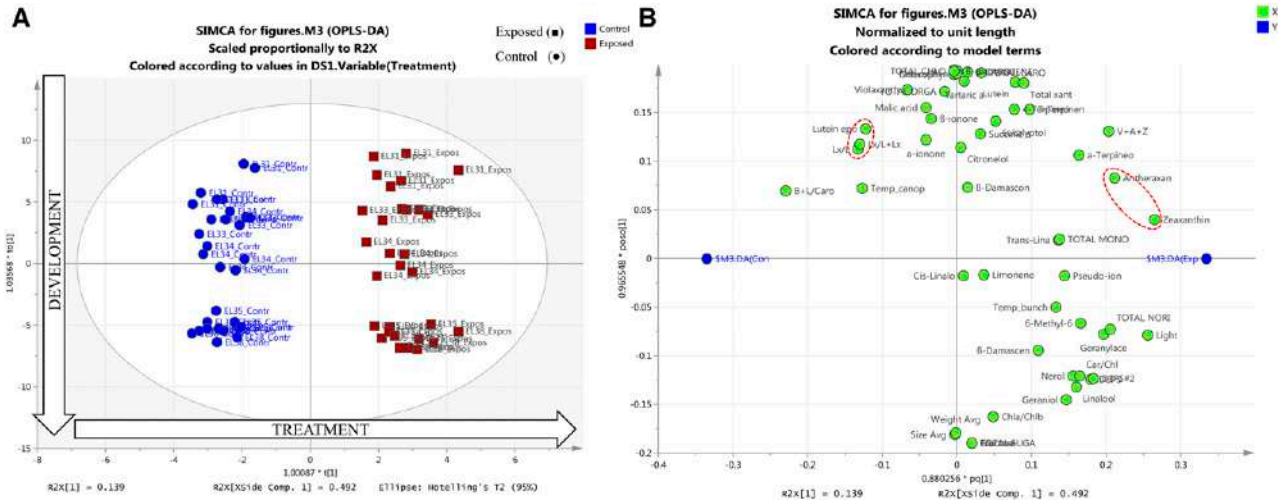


Figure 4. Supervised (treatment) OPLS of all metabolites from all stages. A, Scores plot for the respective samples. Samples are colored by treatment; control samples are indicated by circles and exposed samples by squares. B, Loadings plot for the measured variables in green and discriminant classes/categories in blue. Compounds significantly contributing to the models are circled in red.

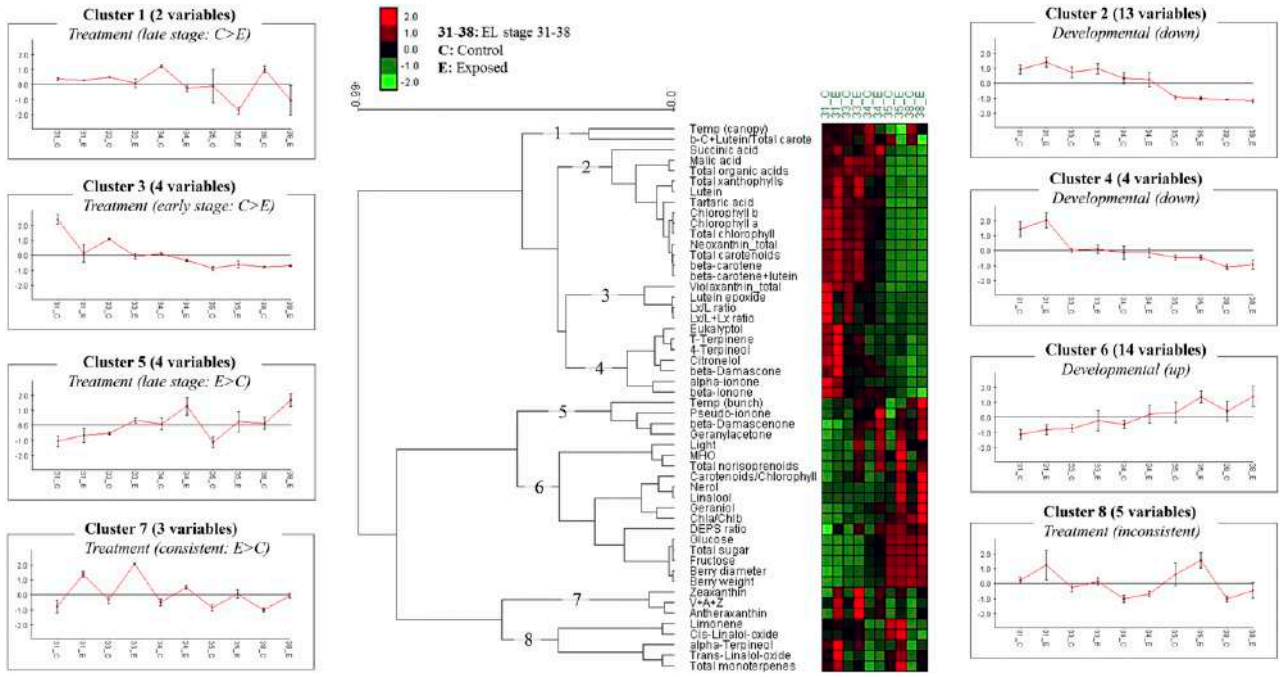


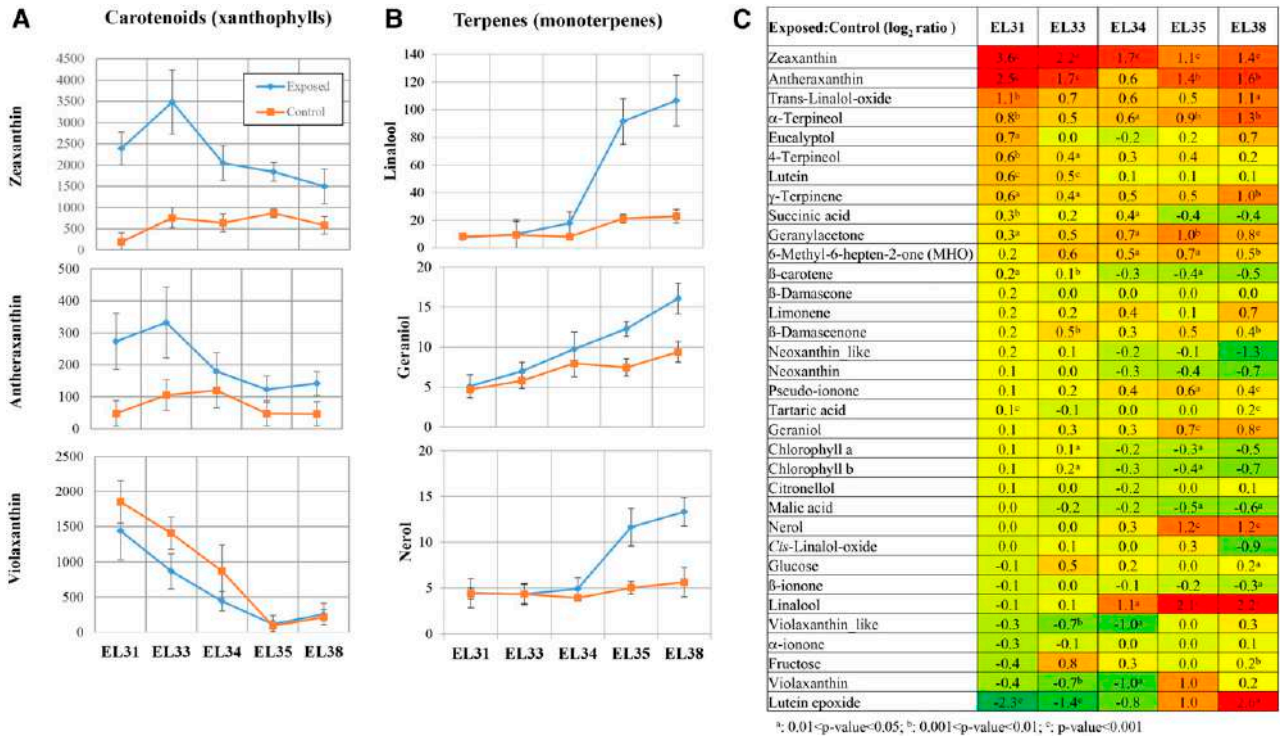
Figure 5. Hierarchical cluster analysis of all variables from all stages, with line graphs of representative clusters.

derived from the hydrolysis of Suc (as expected in a sink organ).

Although the absolute concentrations of the individual organic acids were not significantly affected by the leaf removal treatment across all developmental stages (all but EL38), interesting trends could be seen in the ratio of tartaric acid to malic acid (Supplemental Fig. S6). This ratio, referred to as the β-ratio (proposed by Shiraishi [1995]), has been used previously to evaluate the organic acids from *Vitis* spp. germplasm collections. Until véraison, the β-ratio remained relatively constant (approximately 1) for the exposed and control berries, but from EL35, the ratio increased in both the exposed and control berries. At harvest (EL38), the exposed berries had a β-ratio of 4, double that of the control berries (with a β-ratio of 2). This phenomenon is due to the combination of a slight (but statistically significant) increase in tartaric acid concentrations and a concomitant decrease in malic acid concentrations (relative to the control berries; Supplemental Fig. S4E). Across all stages, the percentage of tartaric acid and malic acid (relative to total organic acids), however, remained relatively constant (approximately 85%–90% of total acids) for both the exposed and control berries. Succinic acid levels were similar in the exposed and control berries and fluctuated from 5% to 15% of total organic acids (Supplemental Fig. S6B). In grapes, malate levels have been shown to be more susceptible to temperature-induced degradation than tartrate, but since the bunch temperatures were not significantly different between the treatments, it is not possible to link bunch temperature to this observation (Sweetman et al., 2014). The canopy temperature of the exposed vines, however, was significantly higher than the control canopy temperature during the night, and it is possible that differences in photorespiration in the leaves, for example, affected the organic acid levels in the berries. The mechanism for this is not known and deserves further investigation.

Major Carotenoids and Chlorophylls Were Predominantly Developmentally Regulated, But the Xanthophylls Responded to the Treatment

Pathway analysis was used to analyze the metabolism of the carotenoids (Fig. 7). For carotenoid metabolism (biosynthesis and catabolism), the pathway described by Young et al. (2012) was used to provide an overview of the relative changes and flux of the related metabolites over time. The regulated catabolism of chlorophylls and the concomitant decrease in total carotenoid concentration are well described for grape berry development (Razungles et al., 1996; Young et al., 2012). The ratio of chlorophyll *a* to chlorophyll *b* increased from 2.5 (EL31) to 3.5 (EL38), with no significant differences between the ratio in exposed berries versus control berries (Supplemental Fig. S7). Until véraison, grapevine berries are photosynthetically active, albeit at much lower levels (1%–10%) than photosynthetically active leaves (Goodwin, 1980). The decrease in the more abundant carotenoids (i.e. lutein and β-carotene, representing approximately 80% of the total carotenes in a grape berry) followed the trends of chlorophylls *a* and *b* in both the control and exposed berries and was generally associated with the developmental stages of berries, with the



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Exposed:Control (log_2 ratio)	EL31	EL33	EL34	EL35	EL38
Zeaxanthin	3.6[c]	2.2[c]	1.7[c]	1.1[c]	1.4[c]
Antheraxanthin	2.5[c]	1.7[c]	0.6	1.4[b]	1.6[b]
Trans-Linalol-oxide	1.1[b]	0.7	0.6	0.5	1.1[a]
α-Terpineol	0.8[b]	0.5	0.6[a]	0.9[b]	1.3[b]
Eucalyptol	0.7[a]	0.0	-0.2	0.2	0.7
4-Terpineol	0.6[b]	0.4[a]	0.3	0.4	0.2
Lutein	0.6[c]	0.5[c]	0.1	0.1	0.1
γ-Terpinene	0.6[a]	0.4[a]	0.5	0.5	1.0[b]
Succinic acid	0.3[b]	0.2	0.4[a]	-0.4	-0.4
Geranylacetone	0.3[a]	0.5	0.7[a]	1.0[b]	0.8[c]
6-Methyl-6-hepten-2-one (MHO)	0.2	0.6	0.5[a]	0.7[a]	0.5[b]
ß-carotene	0.2[a]	0.1[b]	-0.3	-0.4[a]	-0.5
ß-Damascone	0.2	0.0	0.0	0.0	0.0
Limonene	0.2	0.2	0.4	0.1	0.7
ß-Damascenone	0.2	0.5[b]	0.3	0.5	0.4[b]
Neoxanthin_like	0.2	0.1	-0.2	-0.1	-1.3
Neoxanthin	0.1	0.0	-0.3	-0.4	-0.7
Pseudo-ionone	0.1	0.2	0.4	0.6[a]	0.4[c]
Tartaric acid	0.1[c]	-0.1	0.0	0.0	0.2[c]
Geraniol	0.1	0.3	0.3	0.7[c]	0.8[c]
Chlorophyll a	0.1	0.1[a]	-0.2	-0.3[a]	-0.5
Chlorophyll b	0.1	0.2[a]	-0.3	-0.4[a]	-0.7
Citronellol	0.1	0.0	-0.2	0.0	0.1
Malic acid	0.0	-0.2	-0.2	-0.5[a]	-0.6[a]
Nerol	0.0	0.0	0.3	1.2[c]	1.2[c]
Cis-Linalol-oxide	0.0	0.1	0.0	0.3	-0.9
Glucose	-0.1	0.5	0.2	0.0	0.2[a]
ß-ionone	-0.1	0.0	-0.1	-0.2	-0.3[a]
Linalool	-0.1	0.1	1.1[a]	2.1	2.2
Violaxanthin_like	-0.3	-0.7[b]	-1.0[a]	0.0	0.3
α-ionone	-0.3	-0.1	0.0	0.0	0.1
Fructose	-0.4	0.8	0.3	0.0	0.2[b]
Violaxanthin	-0.4	-0.7[b]	-1.0[a]	1.0	0.2
Lutein epoxide	-2.3[c]	-1.4[c]	-0.8	1.0	2.1[c]

[a]: 0.01<p-value<0.05; [b]: 0.001<p-value<0.01; [c]: p-value<0.001

Figure 6. Bar graphs of selected individual carotenoids (A; ng/g FW) and monoterpenes (B; ng/g FW) as well as a heat map (log_2 fold change) representation of all analyzed metabolites (C). FW, Fresh weight.

earlier stages typically having higher concentrations than the later stages (Figs. 5, cluster 2, 6, and 8). The levels of lutein closely followed the trend of chlorophyll *b*, whereas β-carotene followed chlorophyll *a* degradation (Supplemental Fig. S8).

The responses of specific carotenoids, the xanthophylls (i.e. lutein, lutein epoxide, zeaxanthin, antheraxanthin, and violaxanthin), to light are well described in a host of different photosynthetic organisms (for review, see Cunningham and Gantt, 1998; Jahns and Holzwarth, 2012). Of particular importance in this study were the two xanthophyll cycles: (1) the lutein: lutein epoxide cycle and (2) the zeaxanthin:violaxanthin cycle. These two cycles are functional in plants in response to shade and high light, respectively. The lutein: lutein epoxide cycle is considered taxonomically restricted (predominantly woody plants and not formed in Arabidopsis [*Arabidopsis thaliana*], for example), and it has been proposed that it is involved in the maintenance of photosynthetic performance under limiting light as well as serves a photoprotective function, especially in response to sudden changes in irradiance (Esteban et al., 2009). Lutein epoxide typically accumulates in older leaves that are predominantly in the shade but has been reported in grape berries (Razungles et al., 1996; Young et al., 2012).

The levels of lutein epoxide were significantly lower in the berries from exposed vines (relative to the berries from control vines) in the first two stages of development (i.e. EL31 and EL33; Fig. 8). Lutein epoxide displayed the largest coefficient of variation (135% for exposed versus control) of all the metabolites analyzed (Supplemental Fig. S9). The ratio of lutein epoxide to lutein was 10% that of the ratio of berries from control vines in EL31 (Fig. 8). The lutein epoxide-lutein ratio stayed relatively low and constant in the exposed berries but decreased rapidly in the berries from control vines from the initial high at EL31. From stage EL35 onward, the lutein epoxide-lutein ratio was low (less than 0.01) and not significantly different in the berries from exposed vines (relative to the control berries). Lutein epoxide, and to a lesser extent violaxanthin, decreased in the berries from exposed vines (Fig. 8), and conversely, zeaxanthin and antheraxanthin increased in the berries from exposed vines relative to the control. It is also interesting that the ratio of β-carotene to lutein (as an indicator of flux to the β- and α-branches of the carotenoid metabolic pathway) was lower in the exposed berries relative to the control berries. This was due to lower levels of lutein in the control berries (resulting in a higher β-carotene-lutein ratio). The lutein in the control berries was presumably converted to lutein epoxide in the shaded conditions. Conversely, comparatively low levels of lutein epoxide were found in exposed berries (Fig. 8). Although lower levels of lutein were present in the control berries, it still followed a similar developmental pattern to β-carotene and chlorophylls *a* and *b* (Fig. 8), but the linear relationship between lutein and chlorophyll *b* was lower in the control berries than in

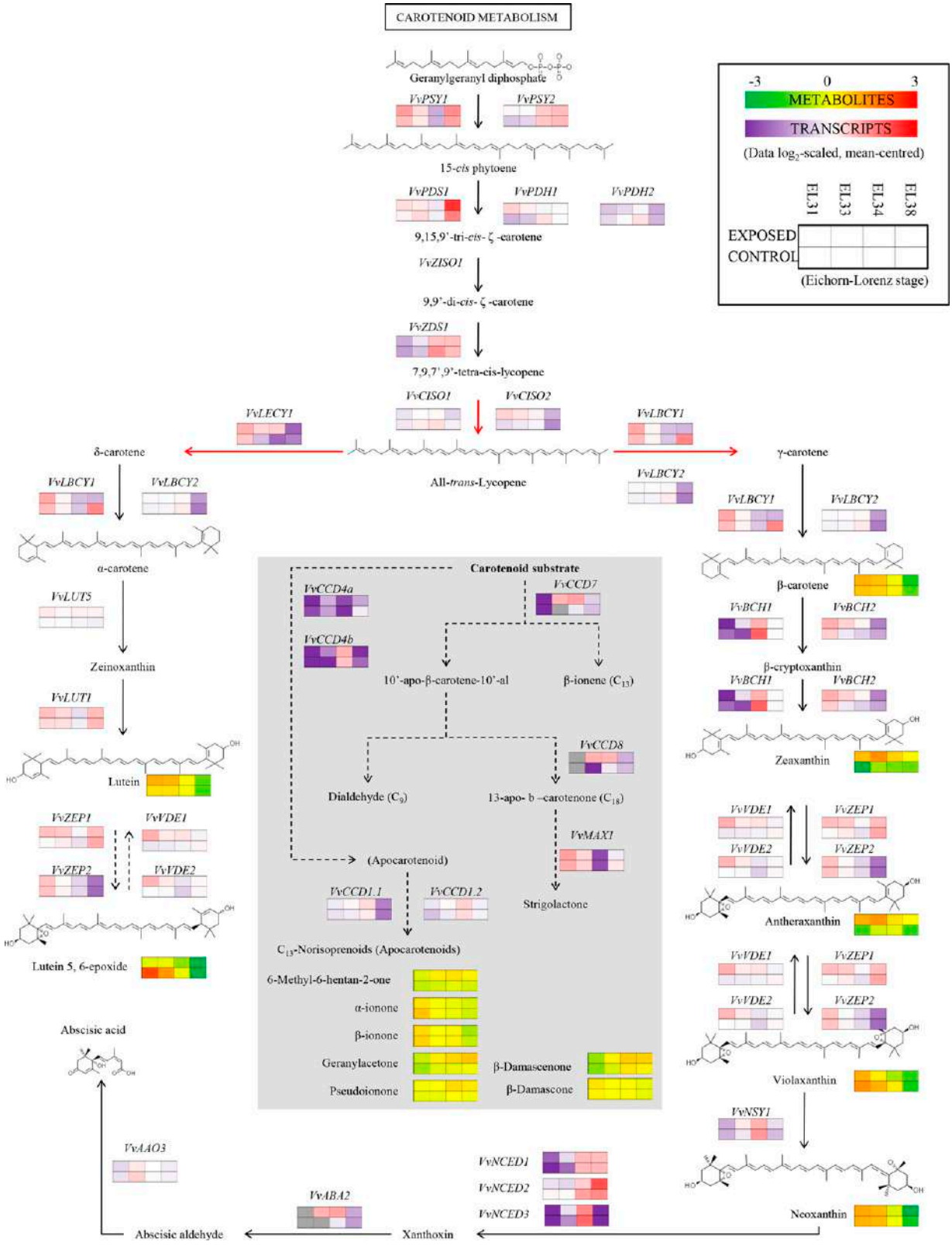


Figure 7. Pathway analysis of genes and enzymes involved in carotenoid metabolism. The heat maps represent the transcript (purple-red) and metabolite (green-red) data (log_2 scaled and mean centered). Reactions that have not been fully elucidated are indicated with dotted lines. Enzymes involved in the branch points in carotenoid metabolism are indicated with red arrows.

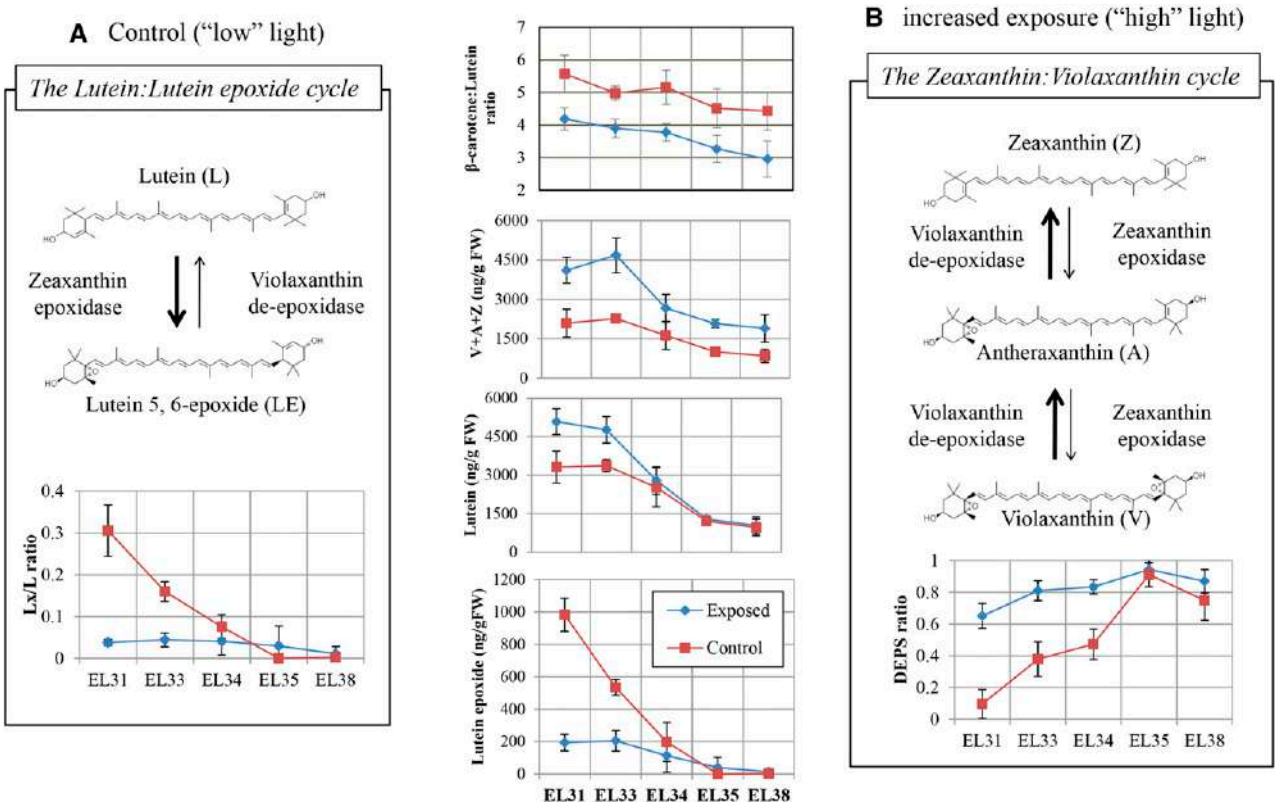


Figure 8. The xanthophyll cycles functional in grapevine and the individual carotenoids involved. A, The lutein:lutein epoxide cycle. FW, Fresh weight. B, The zeaxanthin:violaxanthin cycle. DEPS ratio, The deepoxidation state of the zeaxanthin:violaxanthin cycle [calculated as (Z+E)/(V+A+Z)]; Lx/L, the epoxidation state of the lutein:lutein epoxide cycle (calculated as lutein epoxide/lutein); V+A+Z, violaxanthin, antheraxanthin, and zeaxanthin. Bold arrows indicate increased flux.

the exposed berries (Supplemental Fig. S8). As mentioned, in photosynthetic tissues, a linear relationship was found for major carotenes (β-carotene and lutein) and chlorophylls (chlorophylls *a* and *b*).

The ability to modulate the levels of specific carotenoids by a viticultural treatment is of particular interest, since the carotenoids have been shown to be precursors for the flavor and aroma compounds, the norisoprenoids (apocarotenoids). It has also been shown that carotenoid cleavage dioxygenases catalyze the cleavage of specific C_{40}-carotenoid substrates to specific C_{13}-apocarotenoid cleavage products (Mathieu et al., 2005, 2006; Lashbrooke et al., 2013).

Genes Encoding Specific Xanthophyll Deepoxidation Enzymes, as Well as Branch Point Enzymes in Carotenoid Metabolism, Are Differentially Expressed in Response to the Treatment

In order to determine the contribution of transcriptional regulation to the metabolic plasticity observed in specifically carotenoid and carotenoid-derived metabolites, the transcripts encoding the enzymes involved in carotenoid metabolism were analyzed. Pathway analysis showed that the majority of the genes were not differentially affected by the treatment (across the four developmental stages analyzed for expression: EL31, EL33, EL34, and EL38; Fig. 7). Only five pathway genes were significantly affected by the treatment across the developmental stages ($P \leq 0.05$). The majority of the differentially expressed genes (four of five) were up-regulated in the exposed bunches (versus the control bunches). Three of the up-regulated genes are directly involved in xanthophyll metabolism: *VvVDE1* and *VvVDE2*, encoding violaxanthin deepoxidase that catalyzes the deepoxidation of violaxanthin to zeaxanthin (via antheraxanthin), and *VvLUT5*, a cytochrome P450 gene (CYP97A4) encoding a carotenoid β-ring hydroxylase that catalyzes the conversion of α-carotene to zeinoxanthin and is involved in lutein biosynthesis (Tian and DellaPenna, 2004; Kim et al., 2009). The remaining two differentially affected transcripts encode carotenoid isomerases, *VvCISO1* and *VvCISO2*, and were differentially affected by the treatment. *VvCISO1* was down-regulated in the exposed bunches, whereas *VvCISO2* was up-regulated (Yu et al., 2011).

As was evident in the metabolite data, interesting results can be seen if the developmental stages were analyzed separately (i.e. by treatment per developmental stage). The early developmental stages had the most genes significantly ($P \leq 0.05$) differentially affected by the treatment (exposed versus control

bunches) of the four stages analyzed (Supplemental Table S2). The majority (12 of the 13 genes) in stage EL31 were up-regulated in the exposed bunches (compared with the control bunches), with only *VvBCH1* being down-regulated. *VvBCH1* encodes a β-carotene hydroxylase that catalyzes the hydroxylation of β-carotene (a carotene) to zeaxanthin (a xanthophyll). Conversely, *VvBCH2* is up-regulated. Both *VvVDE1* and *VvVDE2* were similarly up-regulated, as were *VvLUT1* and *VvLUT5*. The net effect of this will hypothetically lead to the accumulation of the deepoxidized xanthophylls lutein and zeaxanthin in the two branches of the carotenoid metabolic pathway in which the violaxanthin and lutein epoxide cycles function (Fig. 8). Flux through the carotenoid pathway should also be increased by the up-regulation of a number of genes involved in the initial reactions of carotenoid biosynthesis: *VvPSY1*, *VvPSY2*, *VvPDS1*, *VvZDS1*, and *VvCISO2* collectively result in lycopene biosynthesis. Lycopene, however, does not accumulate in grape berries and is being converted to predominantly β-carotene and lutein. The up-regulation of *VvCCD1.2* in the exposed berries implicates CCD1 in the maintenance of carotenoid homeostasis in the earlier developmental stages (e.g. EL31; Lashbrooke et al., 2013).

In contrast to the up-regulation of a relatively large number of genes in the early stages of development, the later stages of berry development were characterized by less transcriptional (differential) activity, with the majority of responses being the down-regulation of genes involved in carotenoid catabolism. Of the five transcripts differentially expressed in the exposed versus control berries, only *VvBCH2* was significantly up-regulated at EL38. Of the significantly down-regulated genes, only *VvPSY1* is involved in carotenoid biosynthesis. *VvPSY1* encodes the first dedicated carotenoid biosynthetic enzyme, phytoene synthase. The remaining three genes encode enzymes involved in carotenoid catabolism and were down-regulated: a neoxanthin synthase (*VvNSY1*) and a 9-cis-epoxy carotenoid dioxygenase (*VvNCED2*) involved in abscisic acid metabolism (Frey et al., 2012; Young et al., 2012) and a carotenoid dioxygenase (*VvCCD4a*) involved in C_{13}-norisoprenoid (apocarotenoid) production (Lashbrooke et al., 2013). The decrease in the transcriptional activity of these genes, therefore, followed the overall decrease in their carotenoid substrates.

Volatile Terpenoids Are Increased in Response to Leaf Removal in the Later Stages of Berry Development

The volatile terpenoids measured in this study can be grouped into two major classes: the C_{10}-monoterpenes and the C_{13}-norisoprenoids (or apocarotenoids). The monoterpene content of berries was dominated by the two most abundant monoterpenes: linalool and α-terpineol. The total monoterpene content was affected by the decline in the more abundant linalool in the first three stages (EL31, EL33, and EL34) and then a shift to the increase in α-terpineol in the later developmental stages (EL35 and EL38; Figs. 6B and 9A). A number of monoterpenes were significantly higher in specific stages in the exposed versus the control berries, such as trans-linalool oxide (more than 2-fold in EL31), linalool (more than 2-fold in EL34 and more than 4-fold in EL35), and nerol (more than 2-fold in EL35), but the majority of monoterpenes were typically higher in the exposed berries (versus the control) at harvest (EL38), such as γ-terpinene, trans-linalool oxide, nerol, and α-terpineol (more than 2-fold) and linalool (more than 4-fold; Fig. 6, B and C).

The total volatile norisoprenoids (i.e. α-ionone, β-ionone, pseudo-ionone, geranylacetone, MHO, and β-damascenone) in berries increased until (exposed berries) and EL38 (control berries). MHO and geranylacetone are the two most abundant norisoprenoids, contributing 45% to 60% and 40% to 55%, respectively, to the total norisoprenoid pool in berries. The treatment resulted in higher norisoprenoid content in the exposed berries (relative to the control berries) at the harvest stage (EL38; Figs. 6C, 9, and 10).

Systematic Analysis of the Inherent Variation in the Model Vineyard

Due to the inherent variability of field studies (due to a host of factors), a systematic analysis of the measurable variation between the respective biological repeats (i.e. panels in this study) was undertaken at each sampling time point using all the measured variables (metabolites and microclimatic variables).

Hierarchical cluster analysis of the metabolite concentrations of the samples (per panel) was performed for the entire season and per developmental stages (Supplemental Fig. S10). Based on the variables, hierarchical cluster analysis showed that the separation of the samples across all stages was predominantly on development. Stages EL31, EL33, and EL34 formed a clearly defined early-stage group/cluster, and EL35 and EL38 formed a separate distinct late-stage group/cluster. Within the early stages (EL31–EL35), the samples clustered predominantly by treatment (exposed versus control), whereas in the later stages (EL35 and EL38), the samples clustered predominantly according to their developmental stage and then subclustered within this grouping into their respective treatments (Supplemental Fig. S10). Supplemental Figure S11 shows an unsupervised PCA of the metabolite data of two consecutive seasons (2010-2011 and 2011-2012). Consistent metabolite trends are clear in both years in response to the same leaf removal treatment, showing that, irrespective of vintage, the metabolites showed a consistent response.

DISCUSSION

The field-omics approach provided an analysis of the leaf removal treatment by following metabolite changes during the developmental and ripening stages of the

A

Exposed:Control (Log2 ratio)	EL31	EL33	EL34	EL35	EL38
Total sugars	-0.1	0.6	0.2	0.0	0.2[b]
Total organic acids	0.1[b]	-0.1	-0.1	-0.2	0.0
Total chlorophylls	0.1	0.2[a]	-0.2	-0.3[a]	-0.5
Total carotenoids	0.3[a]	0.3[c]	-0.1	0.0	-0.1
Total xanthophylls	0.5[b]	0.5[c]	0.2	0.5[c]	0.5
Total monoterpenes	0.5[a]	0.2	0.6	0.7[a]	0.9[c]
Total norisoprenoids	0.2[a]	0.5[a]	0.8[a]	0.7[c]	0.6[c]
Chlorophyll a:Chlorophyll b (ratio)	0.0	-0.1	0.0	0.1	0.2[a]
Carotene:Chlorophyll (ratio)	0.2[a]	0.1[b]	0.1[a]	0.3[b]	0.5[c]
β-Carotene+Lutein (B+L)	0.3[a]	0.2[c]	-0.2	-0.3[a]	-0.4
β-carotene+Lutein:Total Carotenoids (ratio)	0.0	-0.1[c]	-0.1[c]	-0.3[c]	-0.3[c]
V+A+Z[1]	1.0[c]	1.0[c]	0.7[b]	1.0[c]	1.2[b]
DEPS (ratio)[2]	2.8[c]	1.1[c]	0.9[c]	0.0	0.2
Lx:L (ratio)[3]	-3.0[c]	-1.9[c]	-0.9		2.1

[a]: 0.01<p-value<0.05; [b]: 0.001<p-value<0.01; [c]: p-value<0.001
[1] Sum of violaxanthin, antheraxanthin and zeaxanthin;
[2] The de-epoxidation state of the zeaxanthin cycle, calculated as: (Z +A)/(V+A+Z)
[3] The epoxidation state of the lutein epoxide cycle, calculated as: Lutein epoxide/Lutein

B

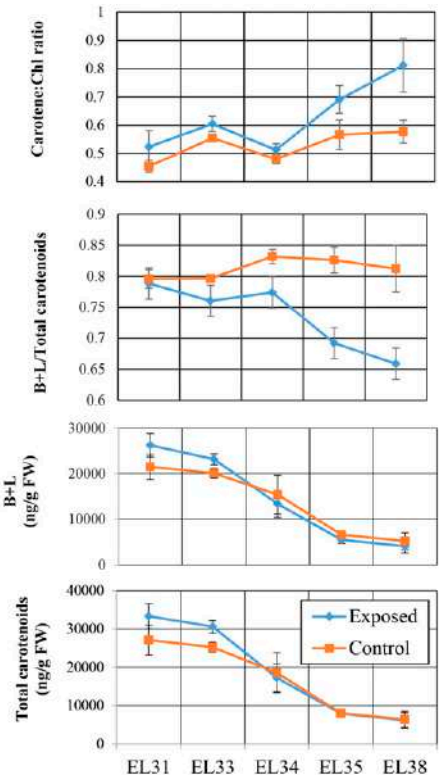
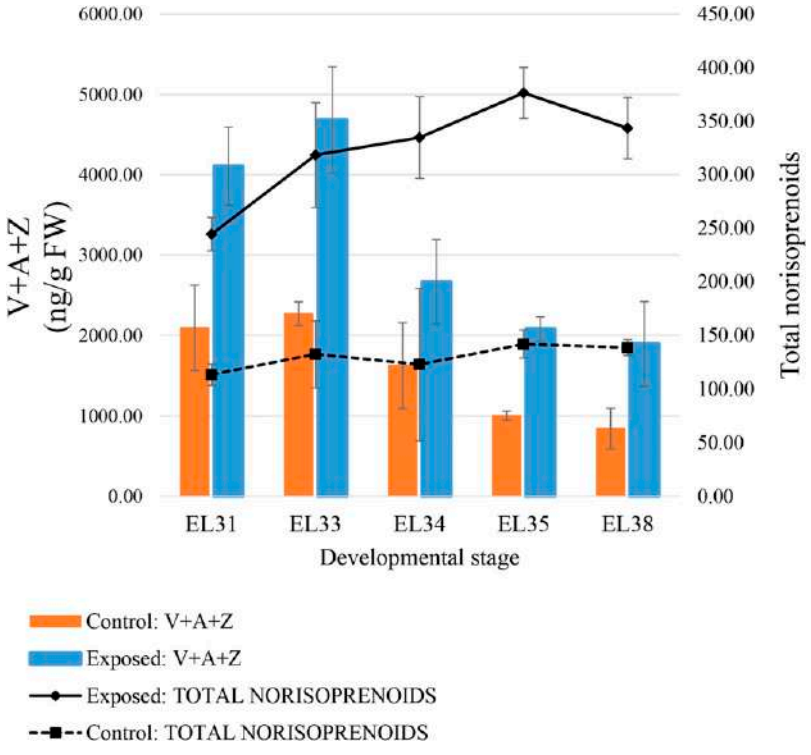


Figure 9. Heat map (log_2 fold change; A) and bar graphs of metabolite pools and selected ratios (per stage; B). FW, Fresh weight.

berry and identified the main berry response to be changes to pigment levels and metabolite pools that have photoprotective and/or antioxidant functions. This logically fits the findings from the environmental profiling that showed an altered (more exposed) microclimate of the treatment. It is possible, of course, that the treatment could have affected other environmental parameters not measured here, but from our

Figure 10. Changes in the norisoprenoid pool and the violaxanthin, antheraxanthin, and zeaxanthin (V+A +Z) pool (in ng/g FW), and in the norisoprenoid pool (in ng/g FW), in the exposed and control berries throughout berry development. FW, Fresh weight.



measurements, statistical analysis confirmed a strong reaction on predominantly light but not bunch temperature.

Compositional Metabolic Plasticity in Grapevine Is Predominantly Due to Stage-Specific Responses in Carotenoids

A number of factors, including variations in the incident light (both quality and quantity), can induce a range of responses that affect plants on multiple levels: from gene transcription to phenotype and from the photosynthetic apparatus to whole-plant architecture. The role of the C_{40}-terpenoid carotenoids in photosynthesis, especially in light harvesting and photoprotection, is well established in numerous photosynthetic organisms, including plant models (for review, see Cunningham and Gantt, 1998). The fate of carotenoids during grape berry development is similarly well documented, with lutein and β-carotene representing the major carotenoids found in grapes (Razungles et al., 1996; Young et al., 2012). The carotenoid concentration in grape berries has been studied in a number of grapevine cultivars, and the total carotenoid levels typically decrease with ripening. Berries until véraison are considered photosynthetically active, carotenes act as light-harvesting antenna pigments, and xanthophylls (oxygenated carotenes) are involved in photoprotection of the plant via the xanthophyll cycles (via lutein:lutein epoxide and zeaxanthin: violaxanthin cycling) in photosynthetic tissues (for review, see Cunningham and Gantt, 1998). Carotenoid concentrations in the grape berries are affected by a number of factors that include the region, the cultivar, exposure to sunlight, and the ripening stage of the berries (Oliveira et al., 2003, 2004; Lee et al., 2007; Song et al., 2015).

From the data presented, it was clear that grapevine berries were capable of more than one response to the altered microclimate. The first response was the modulation of the carotenoid composition in response to the treatment. Most notable was the response of the photoprotective xanthophylls (i.e. zeaxanthin and antheraxanthin; Figs. 6A and 8). Zeaxanthin and antheraxanthin were significantly higher in the exposed berries, and this resulted in a larger xanthophyll pool size (violaxanthin, antheraxanthin, and violaxanthin) and, consequently, an increase in the deepoxidation state of the xanthophylls (deepoxidation state ratio; Fig. 8). Interestingly, the ratio of β-carotene and lutein to the total carotenoid pool remained constant in the control berries but showed a marked decrease in the exposed berries (Fig. 8). Since β-carotene and lutein were unaffected by the treatment (Figs. 6C, 8, and 9), this is due to the total carotenoid pool, especially the xanthophyll pool, increasing in the exposed berries relative to the control berries (Figs. 7–9). Conversely, lutein epoxide levels were significantly lower in the berries from the exposed vines (relative to the berries from control vines; Figs. 7 and 8). The zeaxanthin:violaxanthin cycle is ubiquitous in higher plants, whereas the lutein:lutein epoxide cycle is considered taxonomically restricted, and its occurrence in grapevine berries was only recently shown (Deluc et al., 2009; Crupi et al., 2010b; Young et al., 2012). This resulted in a significantly lower lutein epoxide-lutein ratio in the exposed berries in the early stages (EL31 and EL33; Figs. 7–9). The relationship between lutein epoxide and lutein is markedly different in the exposed and control (shaded) berries (Fig. 8). There is a linear relationship between lutein epoxide and lutein in the exposed berries across the developmental stages (r^2 = 0.98). The relationship between lutein epoxide and lutein in the control berries, however, was not linear (r^2 = 0.75). This could be due to the slow recovery/relaxation of lutein epoxide to lutein in shade conditions, as reported previously (García-Piazola et al., 2007; Esteban et al., 2009; Förster et al., 2011). It is clear that the berries respond to their microclimate utilizing a photoprotective mechanism that is conserved in photosynthetic tissues. Although identified in 1975 in green tomato (*Solanum lycopersicum*) fruit (Rabinowitch et al., 1975), the functionality of the lutein epoxide:lutein cycle in fruit (not leaves) is still relatively unknown. Lutein epoxide has been reported in the petals of flowers (e.g. dandelion [*Taraxacum officinale*]; Meléndez-Martínez et al., 2006) and a minor xanthophyll in squash (*Cucurbita maxima*; Esteban et al., 2009).

Early-Stage-Specific Increases in Carotenoids Result in Concomitant Late-Stage-Specific Increases in Volatile Apocarotenoids

The specific carotenoids formed in grape berries are of particular interest, as their degradation products give rise to the impact odorants, the C_{13}-apocarotenoid/norisoprenoids (Mathieu et al., 2005; Lashbrooke et al., 2013). The norisoprenoids (products) formed are known to be specific to carotenoids, and these degradation products are considered potent varietal flavor and aroma compounds and include α-ionone, β-ionone, pseudo-ionone, geranylacetone, β-damascenone, and vitispirane (Razungles et al., 1996; Baumes et al., 2002; Flamini, 2005; Mendes-Pinto, 2009; Crupi et al., 2010a). Norisoprenoid formation/carotenoid degradation can be catalyzed enzymatically (by the carotenoid cleavage dioxygensase) or physically (by oxidation and/or thermal decomposition; Enzell, 1985; Baldermann et al., 2013).

The increased volatile norisoprenoid concentration in the exposed berries was positively correlated to the increased carotenoid pool (Fig. 10). Previous research has shown that specific carotenoids serve as substrates for carotenoid cleavage dioxygenases, resulting in the formation of volatile C_{13}-norisoprenoids (Mathieu et al., 2005, 2006; Lashbrooke et al., 2013). Lashbrooke et al. (2013) identified and functionally characterized three grapevine carotenoid cleavage dioxygenases (VvCCD1, VvCCD4a, and VvCCD4b). The *VvCCD1*, *VvCCD4a*, and *VvCCD4b* transcripts were detected in all berry developmental stages tested (i.e. green, véraison, and

harvest stages), with *VvCCD4a* having the highest relative expression, peaking at véraison. The different VvCCDs were also shown to have different substrate specificities for their carotenoid substrates and norisoprenoid products formed (Lashbrooke et al., 2013). Here, we have shown an increase in the xanthophyll pool size that potentially serves as a substrate for the chloroplast-localized VvCCD4 enzymes (Figs. 7–9).

From the pathway analysis of carotenoid metabolism (Fig. 7), the expression of the CCD-encoding genes showed interesting differences between the exposed and control bunches: the cytosolic CCD1 was up-regulated in the exposed bunches in the earlier stages of development (from EL31 to EL35/véraison), with *VvCCD10.2* having higher expression levels than *VvCCD10.1*. The cytosolic CCD1 presumably plays an indirect recycling role in maintaining the optimal carotenoid composition in the early berry developmental stages, balancing photosynthesis and photoprotection. Conversely, the chloroplastic CCD4-encoding genes were down-regulated in later stages of development (from EL34 to EL38) in the exposed bunches, *VvCCD4b* typically having higher expression levels than *VvCCD4a*. The increased norisoprenoids, therefore, are not due to increased gene expression (of the CCD4-encoding genes) in the exposed berries but rather due to increased substrate (carotenoid) availability.

The volatile norisoprenoid products were concomitantly increased in the later stages (EL35 and EL38; Fig. 10). With the exception of α-ionone and β-ionone, all the analyzed norisoprenoids (MHO, pseudo-ionone, geranyl acetone, and β-damascenone) were higher in the exposed berries versus the control berries, supporting the findings of Crupi et al. (2010a) linking carotenoids to norisoprenoid content. This analysis also provides evidence of how metabolically interconnected events occurring early (EL31 and EL33) in berry development are: significant changes to photosynthetic pigments carry through to the later stages of berry ripening and, potentially, wine characteristics.

The Monoterpene Pool Is Modulated in the Later Stages of Berry Development in Response to Increased Exposure

The C_{10}-monoterpenes and C_{15}-sesquiterpenes are another class of volatile terpene-derived metabolites that contribute in varying degrees to the flavor and aroma of specific grape cultivars and wine (for review, see Ebeler and Thorngate, 2009). The terpene content of grapes has been well studied in relation to flavor and aroma, predominantly in the aromatic cv Muscat-type varieties. The genome sequence of grapevine (Jaillon et al., 2007) has shown that the genes encoding the enzymes catalyzing the synthesis of these metabolites, the terpene synthases (TPSs), occur in a large overrepresented family in grapevine. Martin et al. (2010) reported 69 predicted TPS-encoding loci in the cv Pinot Noir genome, 39 of which were shown to be functional in in vitro assays.

Volatile monoterpene responses were variable but, collectively, significantly increased in the exposed bunches in the later stages of development (from EL34), with EL38 having double the total monoterpene content (Fig. 9A). Most of the monoterpene levels analyzed were higher in the exposed berries at the later stages of berry development (EL35 and EL38). Linalool, nerol, and α-terpineol were the most significantly affected (Fig. 6B). Only 4-terpineol and cis-linalool oxide decreased with developmental stage, and only cis-linalool oxide was lower in the exposed berries (versus the control) at the harvest stage (EL38; Figs. 5 and 6C). Volatile organic compound (including monoterpenes) emissions are known to increase in response to both biotic (pathogens and herbivory) and abiotic (including temperature and light) stresses (for review, see Muhlemann et al., 2014). In 'Malbec' grapevine, Gil et al. (2013) showed increased monoterpene emissions at the preharvest berry developmental stage, with increased UV-B radiation. Since emissions of volatile terpenoids (monoterpenes [C_{10}] and norisoprenoids [C_{13}]) represent a significant loss of photosynthetic carbon to the plant, it is thought that these compounds must play important physiological and/or ecological roles in the protection of plants from environmental constraints (Loreto and Schnitzler, 2010). It is thought that isoprene (a C_5-hemiterpene) and monoterpenes are capable of stabilizing photosynthetic (chloroplastic) membranes and in so doing protect the photosynthetic apparatus from oxidative damage (Loreto and Schnitzler, 2010). Although the mechanism is controversial and currently not properly understood, the volatile terpenes have been demonstrated to possess antioxidant actions. This coupled with their lipophilic nature implies a potential role in membrane functioning (e.g. stability). Since both carotenoids and monoterpenes were affected by the treatment and both compound groups possess antioxidant activity, one interesting possibility is that the monoterpenes accumulate to compensate for the decrease in carotenoids in the later developmental stages (EL35 and EL38) or that the monoterpenes complement the photoprotection of the carotenoids during abiotic stress conditions (increased light and/or temperature) and in so doing are involved in oxidative stress homeostasis (Carvalho et al., 2015).

Interestingly, Šuklje et al. (2014) also reported elevated levels of linalool in wines made from exposed cv Sauvignon Blanc grapes from the same model vineyard (carotenoids were not analyzed). That study did not evaluate the berries but primarily focused on the wines made from the grapes from the respective treatments. The authors showed that exposed bunches led to an increase in thiols and monoterpenes (most notably linalool) in the resultant wines, which were consequently assigned attributes associated with tropical fruit in sensory evaluation. Conversely, the control wines were assigned green pepper, asparagus, and grassy attributes (Šuklje et al., 2014).

The Physiological Relevance of Compositional Metabolite Changes in Berries in Response to Increased Exposure

Grapevine berries in the early developmental stages respond in the same manner as photosynthetic organs (leaves), albeit at much lower levels. This phenomenon has been reported for a number of crop species, including climacteric and nonclimacteric fleshy fruits (e.g. apples [*Malus domestica*] and grape berries, respectively) as well as dehiscent and indehiscent fruits (e.g. peas [*Pisum sativum*] and cereal grains, respectively), as reviewed by Blanke and Lenz (1989). The data suggest that grape berries possess a pool of carotenoids that are intrinsically linked to photosynthesis (i.e. photosynthesis associated [i.e. β-carotene, lutein, neoxanthin, and to a lesser extent violaxanthin]) and, consequently, decrease during development, in much the same trend as chlorophyll (Fig. 5, cluster 2). There is, however, a second, smaller pool of carotenoids, the xanthophylls (i.e. zeaxanthin, antheraxanthin, and lutein epoxide) with the capacity to respond to the environment by modifying their abundance (e.g. depending on the ambient microclimate). This pool does not follow the developmental degradation trend of chlorophyll or the developmental increase of sugars but instead responds to the microclimate (Figs. 5, clusters 3 and 7, and 8). The individual carotenoids selectively accumulating in response to the higher exposure in exposed bunches were zeaxanthin, antheraxanthin, and lutein, with lutein epoxide accumulating in the less exposed control bunches (Figs. 7–9). For some of the carotenoids (i.e. lutein and lutein epoxide), this response only occurs in the earlier developmental stages (e.g. EL31, EL33, and to a lesser extent EL34) but not in the later stages (e.g. EL35 and EL38; Figs. 5 cluster 3, 7, and 8). The data also show that the increased carotenoid pools from earlier stages result in increased carotenoid-derived norsioprenoids in later berry developmental stages (Fig. 10). This is potentially a way of regulating the carotenoid composition in response to the prevailing/ambient conditions: maintaining photosynthesis under favorable conditions and triggering photoprotection during unfavorable conditions (i.e. shade for lutein epoxide or exposure for zeaxanthin). The temporary shifts in carotenoid pools in response to the microclimate can be subsequently catabolized to volatile C_{13}-norisoprenoids and transported out of the chloroplast, and the carotenoid composition optimal for photosynthesis can then be reestablished (de novo). This is the same metabolism that has been reported in photosynthetic leaf tissue and has been described for Arabidopsis (Lätari et al., 2015) and avocado (*Persea americana*) leaves (Förster et al., 2009).

The CCDs provide potential enzymatic candidates for this regulatory role. They are expressed during berry development, and each has a relatively unique carotenoid substrate specificity, with each carotenoid substrate yielding a different norisoprenoid product (Lashbrooke et al., 2013). Collectively there is a degree of agreement in the up-regulation of genes encoding enzymes involved in flux to carotenoid biosynthesis and the optimal functioning of the xanthophyll (violaxanthin and lutein epoxide) cycles in the earlier developmental stages (i.e. increased zeaxanthin, antheraxanthin, and lutein in the exposed berries). Conversely, the up-regulation of *VvCCD10.2* does not lead to a concomitant increase in the associated C_{13}-apocarotenoids (Fig. 7). The localization of the chloroplastic carotenoids and the cytosolic CCD1 enzyme could be the reason for this disparity, as has been described in Arabidopsis (Auldridge et al., 2006; Floss and Walter, 2009). It is possible that chloroplastic carotenoids are nonenzymatically degraded (due to the treatment) and transported to the cytosol, where they serve as substrates for CCD1. This recycling of carotenoids will ensure that the optimal carotenoid composition is maintained in the chloroplast to either assist photosynthesis or prevent photooxidative damage (Förster et al., 2009).

Although a nonclimacteric fruit, grape berry ripening has been associated with an oxidative burst at the onset of ripening (Pilati et al., 2007, 2014; Rienth et al., 2014). Most stresses result in an oxidative burst, and plants are capable of responding to a diverse array of potentially cooccurring stresses while maintaining active photosynthesis. The up-regulated metabolites described provide metabolite data in support of the hypothesis from a number of grapevine transcriptomic studies (Pilati et al., 2007, 2014; Rienth et al., 2014) that suggests a role in berry oxidative stress homeostasis in ripening grape berries via different antioxidant systems (Carvalho et al., 2015).

We found that the biological basis of the observed phenotypic (metabolic) plasticity is not necessarily in the absolute concentrations of individual metabolites (possibly with the exception of the xanthophylls zeaxanthin and lutein epoxide) but rather the pool size and/or ratio of metabolites within a pool. This is evident in the carotenoid and monoterpene pools and hints at a degree of compensation, possibly linked to their shared antioxidative protective functions (Borges et al., 2014; Kissoudis et al., 2014; Hossain et al., 2015).

We propose that this mechanism of oxidative stress homeostasis then provides the common factor linking the responsive secondary metabolites identified in grapevine leaf removal studies. The biological function of the responsive metabolites is as antioxidants. These include phenolics such as anthocyanins (Neill and Gould, 2003), flavonols (Hernández et al., 2009; Falcone Ferreyra et al., 2012), and stilbenes (for review, see Flamini et al., 2013), ascorbate (Melino et al., 2011), glutathione (Kobayashi et al., 2011), terpenoids (Grassmann, 2005) such as C_{10}-monoterpenoids (Gil et al., 2012), C_{15}-sesquiterpenoids, and C_{40}-tetraterpenoids (for review, see Cunningham and Gantt, 1998), and C_{13}-norisoprenoids (Walter and Strack, 2011). Their presence and associated antioxidant functions, therefore, implicate them in oxidative stress homeostasis observed in ripening grape berries (Pilati et al., 2014; Carvalho et al., 2015) and plant stress responses to, for example, abiotic stresses (Miller et al., 2010; Potters et al., 2010).

CONCLUSION AND FUTURE PROSPECTIVES

The field-omics approach employed in this study showed that the early leaf removal in the bunch zone caused quantifiable and stable responses (over two vintages) in the microclimate, where the main perturbation was increased exposure to light and to a lesser extent temperature, due to the geographical location of the vineyard (high altitude and proximity to the ocean). We showed the physiological impacts on berries in the different developmental stages by studying affected metabolites, providing, to our knowledge for the first time, an explanation for how leaf removal leads to the shifts in grape metabolites typically linked to this treatment (over years). We confirm anecdotal evidence and previous reports that leaf removal treatment at an early stage of berry development affects quality-associated metabolites (monoterpenes and norisoprenoids). Differences in the absolute concentrations of sugars and organic acids were marginal. We show that the main physiological response occurs in the early stages of berry development, when the berry is still photosynthetically active and, therefore, responds to changes to the microclimate in the same way as the major photosynthetically active organs (leaves). This also shows that berries in more shaded conditions activate a different protective system involving the conversion of lutein to lutein epoxide. The compositional changes in the carotenoids in the early stages are carried through to the later stages of berry development (e.g. increased norisoprenoids). This, combined with the increase in monoterpenes observed, implicates redox homeostasis and a degree of plant stress management. This topic has received much attention in grapevine (Carvalho et al., 2015) and in plants in general (Potters et al., 2010; Walter and Strack, 2011; Lätari et al., 2015).

The observation of phenotypic plasticity (metabolic/compositional plasticity) in cv Sauvignon Blanc grape berries, however, does not explain how plasticity is primarily regulated. Analysis specifically of the carotenoid metabolic pathway demonstrated that regulation occurs on both the transcriptional and metabolite levels. Further study of the transcriptome of the berries will provide insights into the transcriptional regulatory networks controlling the observed phenotypic (metabolic) plasticity. It would be interesting to compare the degree of plasticity observed in the transcriptome with that of the metabolome.

MATERIALS AND METHODS

Climatic Classification of the Elgin Model Vineyard Site

The vineyard is located in Elgin within the Overberg region of the Western Cape coastal region of South Africa (34°9′52.19′′S; 19°0′57.48′′E). Climatic classification of the Elgin region and the vineyard site was performed on macroclimatic and mesoclimatic scales according to established climatic indices (Tonietto and Carbonneau, 2004). The Heliothermal Index (Huglin, 1978; Tonietto and Carbonneau, 2004) was calculated for the period October 1 to March 31 (considered to be the biologically relevant period in the Southern Hemisphere) and the Winkler Index from September 1 to March 31. The Cool Night Index was calculated for the final month of ripening in the Southern Hemisphere (February 1–28). Hourly macroclimatic data were collected by the Beaulieu automatic weather station (MCSystems), run by the Institute for Soil, Climate, and Water of the Agricultural Research Council and maintained according to the standards of the World Meteorological Organization (Ehinger, 1993), located 1.55 km east of the experimental site. Hourly mesoclimatic data were collected from a dual-channel internal temperature and relative humidity sensor (MCS 486-TRH logger; MCSystems; maintained by Distell) installed within a Gill screen above the canopy.

Experimental Design, Vineyard/Viticultural Treatments and Management, and Sampling and Sample Processing within a Field-Omics Workflow

Grapevines (*Vitis vinifera* 'Sauvignon Blanc'; clone 316 grafted on 101-14 Mgt) were established in 2004. The vines were planted in a northwest-to-southeast row direction with 2.5-m between-row and 1.8-m in-row spacing. The vines were trellised to a double cordon with a vertical shoot-positioning system and pruned in winter to eight two-bud spurs per running 1 m of cordon. The experimental layout and workflow are outlined in Supplemental Figure S1. The vineyard has a deep shale soil with a high moisture content, so although irrigation was available, the vineyard was managed under dryland conditions, as no water constraints, as determined by stem water potential measurements, were experienced by the vines during the growing season (Supplemental Fig. S12). The treatment involved total leaf and lateral shoot removal in the bunch/fruiting zone (corresponding to removal up to approximately 30–40 cm above the cordon) on the northeast-facing side of the canopy (i.e. the facet of the vine that received morning sunlight exposure in the Southern Hemisphere) at EL29. In the control panels, no leaf removal was performed (Supplemental Fig. S1). The treatment was maintained throughout the season, keeping the fruiting zone exposed through continuous lateral shoot removal. The canopy of the control vines was not manipulated, which resulted in more shaded fruiting zones with reduced exposure. The leaf removal treatment were alternated down two adjacent vineyard rows, creating a checkerboard plot layout with each biological repeat (referred to as a panel) consisting of four consecutive vines (i.e. each row consisted of six panels, and each panel consisted of four healthy consecutive vines; Supplemental Fig. S1). Berry samples were collected (n = 48 berries per sample [i.e. per panel]), with 12 panels per sampling date (representing six exposed and six control panels) at five main phenological stages: green stage (pea-sized berries; EL31), prevéraison (EL33), véraison (EL34), ripening (EL35), and ripe berries at harvest (corresponding to the harvest date; EL38), using a supervised sampling method. The sampling is described as supervised due to the fact that samples were not collected randomly. Bunch positioning within the canopy is typically not uniform; therefore, berries were only sampled from representative bunches from the bunch facet exposed to the outside (northeast facing). All berry samples were collected within 1 h (9–10 AM) on the same day for all five sampling dates. Samples were immediately flash frozen in the field in liquid nitrogen. Seeds were removed, and the frozen tissue was ground in liquid nitrogen and, if not used immediately, stored at −80°C for further analysis.

The experimentation was conducted over three consecutive seasons (2010-2011, 2011-2012, and 2012-2013), but detailed data will only be provided and discussed for the 2010-2011 season. Selected higher order analyses, including supporting data from the additional seasons, will be provided where necessary to confirm repeatability over seasons.

Temperature Measurements

In addition to climatic monitoring to determine the climatic indices, the temperature of the canopy and bunches was monitored on a microclimatic scale. The canopy microclimate was monitored with the use of a dual-channel internal temperature and relative humidity logger (TinyTag TGP-4500; Gemini Dataloggers) and the bunch microclimate via flying lead thermistor probes attached to a dual-channel external temperature logger (TinyTag TGP-4520; Gemini Dataloggers).

Temperature was monitored at two levels, (1) mesoclimatic (i.e. above the canopy; continuously) and (2) microclimatic (i.e. within the canopy and within the bunch zone), using TinyTag data loggers (Gemini Dataloggers) from pea-size stage (EL31) until commercial harvest (EL38). Canopy temperatures were monitored with dual-channel (temperature and relative humidity) data loggers, whereas bunch temperatures were monitored using thermistor flying lead probes connected to a dual-channel external temperature data logger. The thermistor probes were positioned on the surface of the fruit

within representative bunches (for the respective treatments) and within the canopy.

Light Intensity Measurements

PAR was measured between 9:30 and 10:30 AM (before and after berry sampling) with an Accupar ceptometer (model LP-80: Decagon Devices). PAR was measured by positioning the ceptometer parallel to the ground within the bunch zone. Ambient PAR (i.e. full sunlight) was measured before and after each canopy measurement. Relative PAR values were expressed as a ratio relative to the ambient light measurement on the sampling day (i.e. as a percentage relative to full sunlight at the time of sampling).

Midday Stem Water Potential

The water status was determined by measuring the stem water potential according to the method described by Choné (2001) by use of a pressure chamber (Scholander et al., 1965). A single fully expanded, mature leaf per plant was selected for the stem water potential measurements as described by Deloire and Heyns (2011).

Berry Characterization

The weight and diameter for each of the berries sampled per biological repeat (i.e. per panel) were determined before sample processing for metabolite analyses. A sample from each of the six biological repeats per treatment consisted of 48 berries sampled from the exposed facet of a bunch. The 48 berries per sample were weighed individually using a laboratory balance, and the diameters were measured with a digital caliper.

RNA Extraction and Sequencing

Total RNA was extracted from three biological replicates sampled at four developmental stages under both exposed and control conditions according to Reid et al. (2006) from the same deseeded homogenized tissue as described for metabolite analysis. Each of the 24 samples was subjected to DNaseI treatment (Roche) to eliminate contamination with genomic DNA. The concentration and purity of the extracted RNA samples were established using a Nanodrop 2000 spectrophotometer (Thermo Scientific), and the integrity of the samples was confirmed through analysis with the Bioanalyzer Chip RNA 7500 series II (Agilent) according to the manufacturer's instructions. Poly(A) mRNA was prepared for each of the RNA samples and sequenced with an Illumina HiSeq 1000 sequencer according to the supplier's instructions.

Berry Metabolite/Compositional Analyses

Analysis of the Major Sugar and Organic Acid Concentrations in Berries Using Reverse-Phase HPLC

The major sugars and organic acids present in grape berries were extracted from 100 ± 10 mg of frozen, ground berry tissue from the five developmental stages and analyzed by reverse-phase HPLC as described by Eyéghé-Bickong et al. (2012).

Analysis of the Carotenoid and Chlorophyll Concentrations in Berries Using Reverse-Phase Ultra-High-Performance Liquid Chromatography

Carotenoid and chlorophylls were extracted from 250 mg of frozen, ground berry tissue from the five developmental stages as described by Lashbrooke et al. (2010). The analysis of these major pigments was done on a Water Acquity ultra-high-performance liquid chromatography system equipped with a diode array detector. Pigment separation was achieved on a Waters UPLC BEH Shield RP18 (2.1 mm × 100 mm, 1.7 μm) column protected with a Waters UPLC BEH guard cartridge (2.1 mm × 100 mm, 1.7 μm), and the column temperature was set to 20°C. The mobile phases were composed of aqueous 5% acetonitrile in 0.1% (v/v) formic acid (A) and 80%/20% acetonitrile/methanol in 0.1% (v/v/v) formic acid (B). The following gradient program was applied: 0 to 1 min, isocratic 60% B at a flow rate of 0.3 mL min^{-1}; 1 to 12 min, nonlinear (gradient 3) from 60% to 99.8% B at flow rates from 0.3 to 0.5 mL min^{-1}; 12 to 13 min, linear 99.8% to 100% B at a flow rate of 0.5 mL min^{-1}; 13 to 13.1 min, linear 100% to 60% B at flow rates from 0.5 to 0.3 mL min^{-1}; and then for 1.9 min, equilibration with an isocratic 60% B at a flow rate of 0.3 mL min^{-1}. The control of the instrument and the acquisition and processing of the generated data were done using Empower 2 software from Waters, and the injection volume was 5 μL.

The quantification of the major pigments in samples was carried out at 450 nm for xanthophylls and β-carotene, 420 nm for chlorophyll *a*, and 470 nm for chlorophyll *b* using external standard calibration based on standard curves plotted using the peak areas and standard concentrations (in μg mL^{-1}). The concentrations in samples were then normalized to the internal standard amount and the sample fresh weight to obtain the sample amount per berry fresh weight (ng g^{-1} fresh weight). β-Apocarotene was used as the internal standard for all pigments.

Analysis of Berry Volatiles Using Head Space-Solid-Phase Microextraction Gas Chromatography-Mass Spectrometry

Authentic standards for the volatile apocarotenoids (β-damascenone, β-damascone, geranylacetone, α-ionone, β-ionone, pseudo-ionone, and MHO), monoterpenes (eucalyptol, limonene, trans-linalool oxide, cis-linalool oxide, linalool, 4-terpineol, citronellol, nerol, geraniol, fenchone, and α-terpineol), and the internal standard (3-octanol) were purchased from Sigma-Aldrich.

Approximately 500 mg of ground, frozen grapevine berry tissue was weighed into a 20-mL gas chromatography vial, and 2 mL of tartaric acid buffer (2 g L^{-1} tartrate, 2.1 g L^{-1} ascorbic acid, and 0.8 mg L^{-1} sodium azide, pH 3) was added to each vial. The preservatives ascorbic acid and sodium azide (Sigma-Aldrich) were added to the buffer in order to inhibit polyphenol oxidase action and to prevent microbial growth during storage and analysis of the berries, respectively (Flamini and Vedova, 2007). The samples were preincubated for 1 h at 100°C to extract the total volatiles (i.e. the free and bound volatile fractions). If not analyzed immediately, samples were stored at −80°C.

Volatiles were extracted by head space solid-phase microextraction (SPME) using a 50/30-μm divinylbenzene/carboxen/polydimethylsiloxane fiber (gray fiber from Supelco; Barros et al., 2012). Prior to use, the fiber was conditioned at 270°C for 60 min in the gas chromatograph injection port according to the manufacturer's specifications (Supelco).

The samples were equilibrated at 60°C for 5 min in a heating chamber (with constant agitation at 250 rpm). After equilibration, the SPME fiber was inserted through the vial septa and exposed to the sample at 60°C for 30 min with constant agitation at 250 rpm. The bound analytes were thermally desorbed from the fiber in the gas chromatograph injection port. After desorbtion, the fiber was maintained for 20 min in the injection port for cleaning in order to prevent potential carryover between samples.

Gas chromatography analysis was carried out on an Agilent 6890N gas chromatograph coupled to a CTC CombiPal Analytics autosampler and an Agilent 5975B inert XL EI/CI MSD mass spectrometer detector through a transfer line. Analysis was done using an Agilent 122-3263 DB-FF AP capillary column (60 m × 250 μm i.d., 0.5 μm). Desorption of analytes from the SPME fiber was performed in the injection port at 250°C by pulsed splitless mode for 1 min. The purge flow was 1 min at 50 mL min^{-1}. The column operating head pressure was raised from 111 kPa to obtain a pulse pressure of 300 kPa for 1 min. Helium was used as the carrier gas, with a constant flow rate of 1 mL min^{-1}. The oven parameters were as follows: initial temperature of 40°C (2 min), a linear increase to a final temperature of 240°C (at a rate of 5°C min^{-1}), and the temperature was held at 240°C for a final 2 min. The total run time was 44 min. The transfer line temperature was maintained at 250°C.

The mass spectrometry detector was operated in scan and selected ion monitoring modes. The scan parameters were set at mass-to-charge ratio (m/z) ranging from 35 to 350. The dwell time for each ion in a group was set to 100 ms . The software used was MSD ChemStation (G1701-90057; Agilent).

Selected ion monitoring was used to identify compounds according to their elution times and manually integrate their areas. The selected ions monitored were as follows: 3-octanol (internal standard), *m/z* = 83; geranylacetone, *m/z* = 69; eucalyptol, limonene, trans-linalool oxide, γ-terpenene, cis-linalool oxide, linalool, 4-terpineol, α-terpineol, Citronellol, nerol, and geraniol, *m/z* = 93; MHO, *m/z* = 108; β-damascenone, *m/z* = 190; β-damascone, α-ionone, and β-ionone, *m/z* = 177; and pseudo-ionone, *m/z* = 124. The quantification of the volatiles in samples was done using external standard calibration based on standard curves plotted using the peak areas of each standard (total ion count) relative to the peak area of the internal standard versus the standard concentration (μg.L^{-1}) of a nine-point standard dilution series.

Statistical Analyses

Standard statistical analyses were performed using Microsoft Excel (version 14) and Statistica (version 12). Where required, the data statistics (*P* values) were adjusted for false discovery rate by Benjamini-Hochberg correction (adjusted *P* values or *Q* values) as described by Trapnell et al. (2012). Hierarchical cluster analysis of metabolites was performed using Expander (Sharan et al., 2003). All multivariate data analyses were performed using SIMCA (version 13.0.3.0 from Umetrics). For multivariate data analysis, data were normalized and analyzed using PCA and/or OPLS-DA. OPLS-DA was used to analyze the quantitative relationship between the data matrix, *x* (i.e. the variables measured [e.g. metabolite concentration and/or transcript levels]), and a vector, *y*, containing qualitative values (e.g. developmental stages [EL31–EL38] or treatment [control/exposed]).

The reads generated from the RNA sequencing were aligned to the grapevine reference genome (X12) using TopHat (version 2.0; Trapnell et al., 2009). Cufflinks (version 2.0) was subsequently used to assemble transcripts from the generated sequence reads (Trapnell et al., 2010). CuffDiff (version 2.0) was used for differential expression analysis between treatments and/or subsequent developmental stages (Trapnell et al., 2010). The putative carotenoid metabolic genes were obtained from Young et al. (2012).

The data reported (i.e. metabolite and expression data) are provided in Supplemental Table S2A as averages ± SD.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Plot layout and field-omics workflow.

Supplemental Figure S2. Mean seasonal data, where different letters indicate significant differences between treatments ($P \leq 0.05$).

Supplemental Figure S3. Berry characterization.

Supplemental Figure S4. Berry characterization: concentration of the major sugars in grapevine berries.

Supplemental Figure S5. Unsupervised PCA of all variables from the study for all developmental stages.

Supplemental Figure S6. Berry characterization: organic acids.

Supplemental Figure S7. Chlorophyll *a*-chlorophyll *b* ratio in developing berries.

Supplemental Figure S8. Relationship between chlorophyll and the major carotenes.

Supplemental Figure S9. ANOVA of developmental stage × treatment of metabolites showing the highest coefficient of variation.

Supplemental Figure S10. Field-omics: assessment of all late variables for all biological repeats (panels 1–6) across all stages (EL31, EL33, EL34, EL35, and EL38) for the 2010-2011 season.

Supplemental Figure S11. Repeatability of the experiment for two consecutive seasons (2010-2011 and 2011-2012).

Supplemental Figure S12. Midday stem water potential at three developmental stages.

Supplemental Table S1. Climatic indices used to classify the Elgin region.

Supplemental Table S2. Mean and SD of the transcripts ($n = 3$) and metabolites ($n = 6$) reported in this study and significance testing (Student's *t* test) of transcripts and metabolites in the various developmental stages (EL31, EL33, EL34, and EL38).

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Addendum C to Chapter 3

This Addendum contains relevant and additional data not shown in Chapter 3.

Table S1: Primers used for Real-time PCR

Gene accession	Grimplet Annotation	Primer sequence (5')	Primer sequence (3')	Amplicon length (bp)	Optimal Ta (°C)
VIT_10s0116g00410	gibberellin 2-beta-dioxygenase 7	AGCCAGTTATCGTGGTCCGAAG	TTGTCCCATTGTGGAGCTGAGG	96	63.9615
VIT_18s0001g03470	Flavonol synthase Vitis vinifera	TCCATAACATCTGGCCTCCTCCTG	TCGTTCGCAGCTCTGTAAGTAGG	79	60.6532
VIT_05s0020g04110	ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN)	TGACACGCGTAGCCAACAGAAC	TCGATTTGGCCATCCTCCTTGG	115	65.3547
VIT_02s0025g04060	Rab/Ypt GTPase Ara4-interacting protein	CAGTGAAGGAGATCGAACCATTGC	GCCATATCAATCATGTCCGTGAGC	69	60.9634
VIT_01s0010g03620	LHCA2 (Photosystem I light harvesting complex gene 2)	TTGCCACCATCTCTCCCAGTTC	CCGTGAACTTCTTCAGGCTCAGTC	102	63.5269
VIT_19s0014g00160	LHCII type I CAB-1	TGGACCGCTAGGAGAAGTGAAG	ACCCGAACATGGAGAACATAGCC	65	59.5942

Table S2: A table summarizing the retention times of phenolic compounds measured

	Standards	Rt (min)	Range (mg/L)	Slope	y-Intercept	r2	LOD (g/L)	LOQ (g/L)
Flavan-3-ols	Catechin	15.17	263-0	11377.214	-24.146	0.99816	0.016	0.054
	Epicatechin	22.45	107-0	14006.536	-63.675	0.99786	0.009	0.029
Phenolic acids	Caftaric acid	13.36	125-0	42534.569	-5.3	0.99976	0.003	0.008
	Caffeic acid	17.4	480-0	102285.985	207.618	0.99938	0.015	0.049
Flavonols	Quercetin-Glucoside	40.41	125-1	45538.719	-19.845	0.99773	0.005	0.014

Table S3: Summary of RNASeq reads and mapping.

	EL31		EL33		EL35		EL38	
Treatment	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
No. of total reads	100712837	101310946	130713888	101080327	81231782	77648208	84861214	86362444
Average overall mapping rate (%)	88.1	88.2	87.8	87.9	87.2	88	87.1	86.6
Number of trancripts expressed (VIT_)	22770	22681	22647	22206	21484	21612	21417	22001
Number of trancripts represented in V1	20995	20905	20834	20495	19897	20030	19819	20297

			EL31						EL33						EL35						EL38					
			Exposed			Control			Exposed			Control			Exposed			Control			Exposed			Control		
			Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	Rep_3	Rep_1	Rep_2	Rep_3
EL31	Exposed	Rep_1	1.000	0.959	0.965	0.964	0.963	0.961	0.939	0.943	0.942	0.942	0.941	0.939	0.905	0.913	0.902	0.911	0.904	0.911	0.862	0.862	0.850	0.864	0.863	*0.942*
		Rep_2	0.959	1.000	0.966	0.957	0.960	0.964	0.937	0.946	0.945	0.944	0.944	0.944	0.907	0.914	0.905	0.910	0.906	0.912	0.863	0.866	0.855	0.865	0.865	*0.946*
		Rep_3	0.965	0.966	1.000	0.961	0.965	0.967	0.944	0.950	0.949	0.951	0.951	0.949	0.913	0.919	0.910	0.918	0.910	0.916	0.867	0.870	0.859	0.869	0.871	*0.950*
	Control	Rep_1	0.964	0.957	0.961	1.000	0.968	0.964	0.934	0.936	0.936	0.941	0.942	0.938	0.901	0.906	0.897	0.912	0.904	0.910	0.859	0.859	0.848	0.862	0.864	*0.940*
		Rep_2	0.963	0.960	0.965	0.968	1.000	0.967	0.933	0.943	0.940	0.945	0.947	0.945	0.907	0.912	0.901	0.917	0.909	0.914	0.865	0.866	0.853	0.867	0.868	*0.947*
		Rep_3	0.961	0.964	0.967	0.964	0.967	1.000	0.939	0.943	0.945	0.948	0.948	0.948	0.907	0.913	0.907	0.914	0.911	0.916	0.864	0.865	0.854	0.867	0.869	*0.946*
EL33	Exposed	Rep_1	0.939	0.937	0.944	0.934	0.933	0.939	1.000	0.959	0.966	0.962	0.961	0.960	0.943	0.948	0.943	0.945	0.941	0.944	0.903	0.904	0.894	0.905	0.905	*0.959*
		Rep_2	0.943	0.946	0.950	0.936	0.943	0.943	0.959	1.000	0.965	0.959	0.965	0.963	0.938	0.941	0.929	0.939	0.928	0.936	0.896	0.899	0.893	0.899	0.899	*0.973*
		Rep_3	0.942	0.945	0.949	0.936	0.940	0.945	0.966	0.965	1.000	0.961	0.966	0.963	0.947	0.950	0.947	0.949	0.944	0.948	0.906	0.907	0.900	0.908	0.909	*0.967*
	Control	Rep_1	0.942	0.944	0.951	0.941	0.945	0.948	0.962	0.959	0.961	1.000	0.966	0.965	0.932	0.938	0.933	0.938	0.930	0.933	0.897	0.895	0.886	0.896	0.899	*0.962*
		Rep_2	0.941	0.944	0.951	0.942	0.947	0.948	0.961	0.965	0.966	0.966	1.000	0.965	0.935	0.937	0.933	0.942	0.933	0.940	0.899	0.902	0.892	0.900	0.904	*0.971*
		Rep_3	0.939	0.944	0.949	0.938	0.945	0.948	0.960	0.963	0.963	0.965	0.965	1.000	0.935	0.938	0.933	0.940	0.935	0.940	0.897	0.900	0.890	0.900	0.901	*0.967*
EL35	Exposed	Rep_1	0.905	0.907	0.913	0.901	0.907	0.907	0.943	0.938	0.947	0.932	0.935	0.935	1.000	0.966	0.964	0.968	0.961	0.966	0.936	0.934	0.932	0.940	0.938	*0.941*
		Rep_2	0.913	0.914	0.919	0.906	0.912	0.913	0.948	0.941	0.950	0.938	0.937	0.938	0.966	1.000	0.961	0.960	0.955	0.960	0.924	0.923	0.917	0.927	0.924	*0.942*
		Rep_3	0.902	0.905	0.910	0.897	0.901	0.907	0.943	0.929	0.947	0.933	0.933	0.933	0.964	0.961	1.000	0.960	0.958	0.962	0.931	0.934	0.927	0.934	0.936	*0.932*
	Control	Rep_1	0.911	0.910	0.918	0.912	0.917	0.914	0.945	0.939	0.949	0.938	0.942	0.940	0.968	0.960	0.960	1.000	0.963	0.968	0.931	0.930	0.922	0.935	0.934	*0.946*
		Rep_2	0.904	0.906	0.910	0.904	0.909	0.911	0.941	0.928	0.944	0.930	0.933	0.935	0.961	0.955	0.958	0.963	1.000	0.966	0.925	0.927	0.918	0.931	0.930	*0.935*
		Rep_3	0.911	0.912	0.916	0.910	0.914	0.916	0.944	0.936	0.948	0.933	0.940	0.940	0.966	0.960	0.962	0.968	0.966	1.000	0.930	0.931	0.924	0.935	0.936	*0.942*
EL38	Exposed	Rep_1	0.862	0.863	0.867	0.859	0.865	0.864	0.903	0.896	0.906	0.897	0.899	0.897	0.936	0.924	0.931	0.931	0.925	0.930	1.000	0.967	0.963	0.965	0.961	*0.902*
		Rep_2	0.862	0.866	0.870	0.859	0.866	0.865	0.904	0.899	0.907	0.895	0.902	0.900	0.934	0.923	0.934	0.930	0.927	0.931	0.967	1.000	0.966	0.963	0.961	*0.903*
		Rep_3	0.850	0.855	0.859	0.848	0.853	0.854	0.894	0.893	0.900	0.886	0.892	0.890	0.932	0.917	0.927	0.922	0.918	0.924	0.963	0.966	1.000	0.962	0.958	*0.897*
	Control	Rep_1	0.864	0.865	0.869	0.862	0.867	0.867	0.905	0.899	0.908	0.896	0.900	0.900	0.940	0.927	0.934	0.935	0.931	0.935	0.965	0.963	0.962	1.000	0.965	*0.904*
		Rep_2	0.863	0.865	0.871	0.864	0.868	0.869	0.905	0.899	0.909	0.899	0.904	0.901	0.938	0.924	0.936	0.934	0.930	0.936	0.961	0.961	0.958	0.965	1.000	*0.904*
		Rep_3	*0.942*	*0.946*	*0.950*	*0.940*	*0.947*	*0.946*	*0.959*	*0.973*	*0.967*	*0.962*	*0.971*	*0.967*	*0.941*	*0.942*	*0.932*	*0.946*	*0.935*	*0.942*	*0.902*	*0.903*	*0.897*	*0.904*	*0.904*	*1.000*

Figure S1: Pearson correlation matrix representing the entire transcriptomes of the initial 24 samples representing 3 biological replicates from control and exposed grapes at four phenological stages.

Table S4: List of positive and negative molecular biomarkers separating green (EL31 and EL33) from ripening (EL35 and EL38) berries (Available online at https://doi.org/10.3389/fpls.2017.01261).

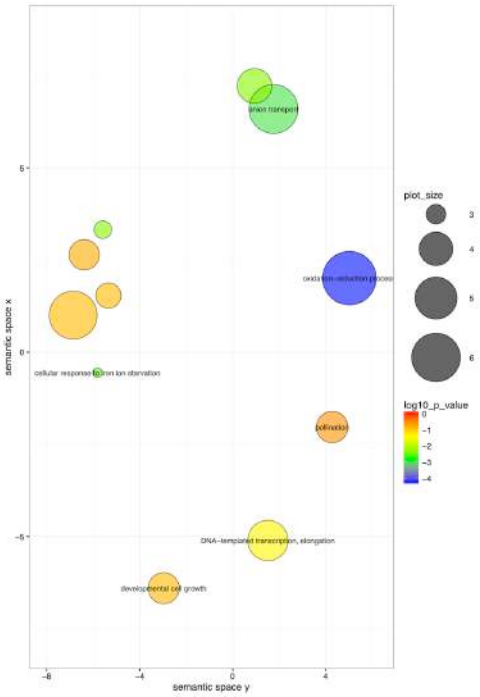


Figure S2: ReviGO analysis output of GO enrichment data generated from the 5050 genes in the grapevine genome that was not expressed whatsoever in the grapes investigated in this study.

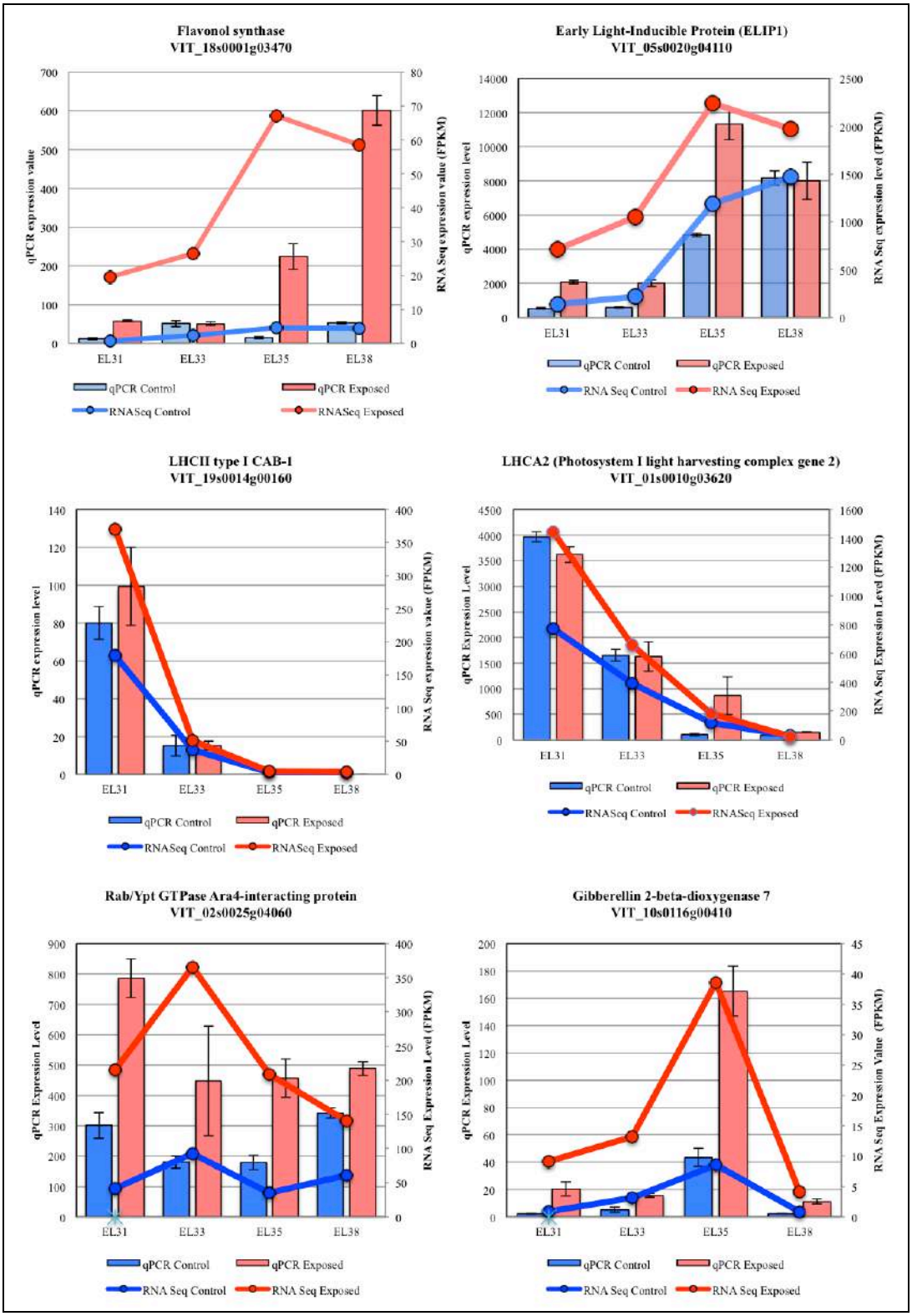
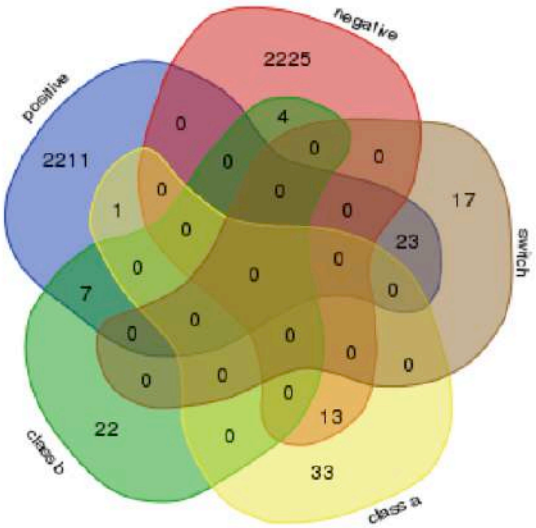


Figure S3: Summarized results generated from Real-time PCR analysis.



		Significantly different (q<0.05) between exposed and control					
Gene ID	Functional Annotation (12X)	EL31	EL33	EL35	EL38	Shared with	Unaffected
VIT_07s0005g01680	stachyose synthase					Switch	Always
VIT_14s0060g00420	pyruvate dehydrogenase kinase					Switch	Always
VIT_11s0118g00580	unknown					Switch	Always
VIT_07s0005g03010	triacylglycerol lipase					Class b	Always
VIT_13s0067g03360	adenylate kinase					Class b	Always
VIT_08s0007g06530	DnaJ homolog, subfamily B, member 6					Class b	Always
VIT_01s0011g00610	Unknown protein					Class a	Always
VIT_01s0146g00070	11-beta-hydroxysteroid dehydrogenase					Class a	Always
VIT_10s0003g04390	ABC transporter C member 11					Class a	Always
VIT_06s0004g00850	DEAD box RNA helicase					Class b	Always
VIT_14s0066g02350	lysyl-tRNA synthetase					Class b	Always
VIT_16s0100g00570	dehydration-responsive protein			*0.02*		Switch	Green stages
VIT_08s0040g01950	zinc finger (C3HC4-type RING finger)			*0.00*		Switch	Green stages
VIT_14s0066g01190	Unknown protein			*0.01*		Switch	Green stages
VIT_15s0048g02280	NAC transcription factor-like 9			*0.01*		Class a	Green stages
VIT_08s0058g00410	ferritin 1 (FER1)				*0.01*	Switch	Green stages
VIT_01s0150g00040	Unknown protein				*0.00*	Class b	Green stages
VIT_09s0002g00980	proteasome 20S alpha subunit A1				*0.02*	Class b	Green stages
VIT_12s0028g03380	ADP,ATP carrier protein				*0.02*	Class a	Green stages
VIT_03s0017g01210	Phosphate-induced protein 1				*0.00*	Class a	Green stages
VIT_16s0022g00670	Vacuolar invertase 1, GIN1				*0.01*	Class a	Green stages
VIT_03s0180g00130	D-3-phosphoglycerate dehydrogenase, chloroplast precursor				*0.00*	Class b	Green stages
VIT_06s0004g00620	ACCELERATED CELL DEATH 1 ACD1	*0.01*				Class a	Ripening stages
VIT_15s0046g02910	Ribosomal protein L21, chloroplast / CL21 (RPL21) 50S	*0.00*				Class a	Ripening stages
VIT_06s0004g03910	Unknown protein		*0.00*			Switch	Ripening stages
VIT_02s0087g00930	9-cis-epoxycarotenoid dioxygenase		*0.00*			Switch	Ripening stages
VIT_01s0127g00680	SRO2 (SIMILAR TO RCD ONE 2)		*0.00*			Switch	Ripening stages
VIT_19s0027g00230	NAC domain-containing protein 22		*0.00*			Switch	Ripening stages
VIT_04s0044g01230	no hit		*0.00*			Switch	Ripening stages
VIT_02s0025g04340	osmotin		*0.00*			Switch	Ripening stages
VIT_08s0040g01200	short-chain type alcohol dehydrogenase		*0.00*			Switch	Ripening stages
VIT_00s0181g00080	F-box domain containing protein		*0.00*			Class b	Ripening stages
VIT_08s0007g08840	Glycosyl transferaseHGA1	*0.01*	*0.00*			Switch	Ripening stages
VIT_16s0050g00390	4-coumarate-CoA ligase	*0.00*	*0.00*			Switch	Ripening stages
VIT_09s0002g06420	lactoylglutathione lyase	*0.01*	*0.00*			Switch	Ripening stages
VIT_14s0219g00040	zinc finger (C3HC4-type RING finger)	*0.02*	*0.00*			Switch	Ripening stages
VIT_08s0007g07620	thioredoxin 2	*0.00*	*0.00*			Class a	Ripening stages
VIT_18s0089g01390	Unknown protein	*0.00*	*0.01*			Class a	Ripening stages
VIT_14s0068g01950	Ribosomal protein L27, chloroplast (RPL27) 50S	*0.00*	*0.00*			Class a	Ripening stages
VIT_16s0098g01150	Auxin-responsive SAUR29		*0.00*	*0.00*		Switch	
VIT_14s0108g00450	no hit		*0.00*	*0.02*		Switch	
VIT_14s0066g01030	Calmodulin-binding heat-shock protein		*0.00*	*0.00*		Class b	
VIT_12s0028g01080	Photosystem II oxygen-evolving complex precursor, 23kda PSBP	*0.00*	*0.00*	*0.00*		Class a	
VIT_05s0020g03180	Photosystem I reaction center subunit II, chloroplast precursor	*0.00*			*0.00*	Class a	
VIT_08s0058g00440	ferritin		*0.00*		*0.01*	Switch	
VIT_14s0068g01760	Alcohol dehydrogenase		*0.00*	*0.01*	*0.00*	Switch	
VIT_00s0214g00090	F-box protein PP2-B10 (Protein PHLOEM PROTEIN 2-LIKE B10)		*0.00*	*0.00*	*0.00*	Switch	
VIT_16s0050g00750	short-chain dehydrogenase/reductase	*0.00*	*0.00*	*0.00*	*0.00*	Class b	

Figure S4: Venn diagram comparing the molecular biomarkers generated in this study to previously published biomarkers from Zamboni et al., (2010) and Palumbo et al., (2014) and differential expression analysis of biomarkers shared between this investigation and previously published biomarkers

Table S5: Table listing the genes most significantly up and downregulated at each developmental stage (-2>Log_2FC>2)(Available online at https://doi.org/10.3389/fpls.2017.01261).

Table S6: Table listing all significantly differentially expressed genes ($q \leq 0.05$; $1.5 \leq Log_2FC \leq -1.5$) significantly correlated to predetermined gene expression clusters according to STEM analysis (Available online at https://doi.org/10.3389/fpls.2017.01261).

Table S7: Functional annotation (Grimplet et al., 2012) of each of the genes that were highly upregulated ($2 \leq Log_2FC \leq -2$) between two or more phenological stages indicated in color as represented in Figure 5. *Q*-values represent the level of significant difference between the expression of each indicated gene at the specific developmental stage. Asterisks (*) indicate multiple genes represented by the same functional annotation with *Q*-values in this case indicative of the average value of the multiple genes sharing the same function.

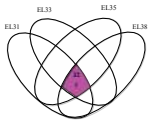
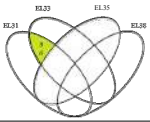
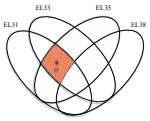
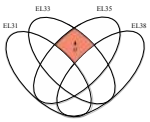
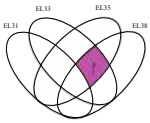
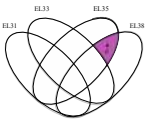
Phenological stages	Functional annotation	*Q*-value			
		EL31	EL33	EL35	EL38
	Chaperone	<0.001	<0.001	<0.001	0.042
	Flavonol synthase	<0.001	<0.001	<0.001	<0.001
	Galactinol synthase	<0.001	<0.001	<0.001	<0.001
	Gibberellin 2-beta-dioxygenase 7	<0.001	<0.001	<0.001	<0.001
	Glycosyl transferase family 8 protein	<0.001	<0.001	<0.001	<0.001
	Heat shock protein 17*	<0.001	<0.001	<0.001	<0.001
	Heat shock protein 18*	<0.001	<0.001	<0.001	<0.001
	Heat shock protein 21	<0.001	<0.001	<0.001	<0.001
	Heat shock protein 23*	<0.001	<0.001	<0.001	<0.001
	Heat shock protein 70	<0.001	<0.001	<0.001	<0.001
	Heat shock protein*	<0.001	<0.001	<0.001	<0.001
	Steroid 5alpha-reductase	<0.001	<0.001	<0.001	0.007
	ELIP1 (Early light inducible protein)	<0.001	<0.001	<0.001	0.016
	Heat shock protein 18	0.008	<0.001	0.268	0.351
	Calmodulin	<0.001	<0.001	<0.001	<0.001
	Heat shock protein 17*	<0.001	<0.001	<0.001	<0.001
	Heat shock protein 18	<0.001	<0.001	<0.001	<0.001
	Heat shock protein 20	<0.001	<0.001	<0.001	<0.001
	Heat shock protein 17	0.055	<0.001	<0.001	0.091
	Seed maturation protein PM31	0.233	<0.001	<0.001	0.005
	Exocyst subunit EXO70 H2	<0.001	<0.001	<0.001	<0.001
	Ubiquitin-conjugating enzyme E2 variant	0.233	<0.001	<0.001	0.141
	Heat shock protein 21	<0.001	<0.001	<0.001	0.005
	FtsH protease	0.322	<0.001	<0.001	<0.001
	Heat shock protein 17	0.082	<0.001	<0.001	<0.001
	Pentatricopeptide (PPR) repeat-containing	*0.277*	*0.004*	*0.015*	*0.007*
	Serine protease inhibitor	1.000	1.000	0.004	0.007
	Aminopeptidase	1.000	1.000	0.004	0.012
	Heat shock protein 16.9	1.000	0.316	<0.001	0.036
	ATAN11 (ANTHOCYANIN11)	0.081	0.829	0.003	<0.001

Table S8: The amino acid concentrations of all the exposed and control grapes sampled from EL31, EL33, EL35 and EL38. D.N.Q. refers to AA concentrations that were detected but were at concentrations below the limit of quantification (Available online at https://doi.org/10.3389/fpls.2017.01261).

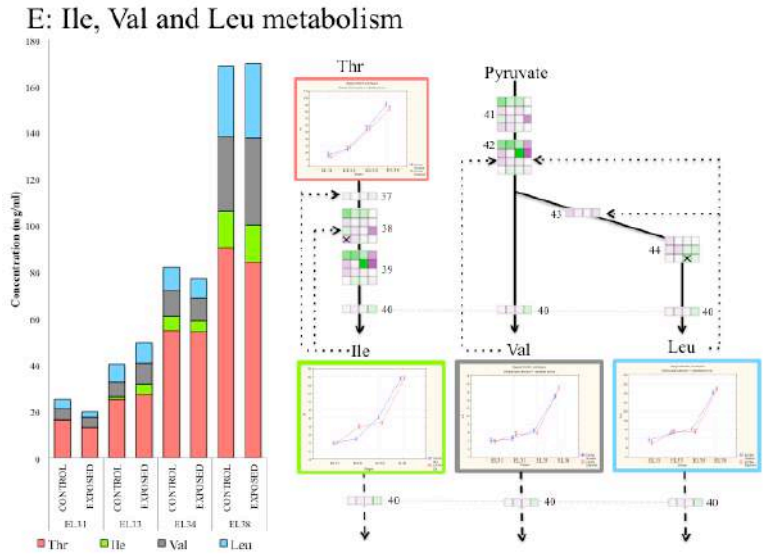


Figure S5: The amino acid super pathway of Ile, Val and Leu biosynthesis.

Table S9: Genes involved in amino acid synthesis and catabolism as indicated by the numbers assigned in Figure 7.

	Number in figure 7	Gene accession	Gene annotation (X12)	Log2 Fold change (Exposed vs Control)			
				EL31	EL33	EL35	EL38
Pathway A: Gly synthesis	1	VIT_05s0029g00310	Serine hydroxymethyltransferase 1	-0.130222	0.0757074	-0.0624502	0.27671
	1	VIT_04s0008g00770	Glycine hydroxymethyltransferase	0.165437	-0.0921235	0.0941349	0.108233
	1	VIT_18s0001g04340	glycine hydroxymethyltransferase	***0.294264***	0.268332	0.203467	0.216408
	1	VIT_00s0211g00120	Glycine hydroxymethyltransferase	***1.87427***	***0.675811***	***0.707631***	***0.45407***
	1	VIT_18s0001g07960	Serine hydroxymethyltransferase	-0.148592	-0.189107	-0.0861917	0.24515
	1	VIT_12s0034g02380	glycine hydroxymethyltransferase	-0.0313181	-0.00926173	0.0007782	0.100461
	1	VIT_00s0211g00080	Serine hydroxymethyltransferase 2	0.037105	-0.0776432	0.380595	0.536548
	1	VIT_06s0009g03740	serine-glyoxylate aminotransferase	***1.65717***	***0.789528***	0.0707028	0.319804
	1	VIT_00s0225g00130	Alanine transaminase.	***0.646075***	***0.325***	0.051118	0.0699332
	1	VIT_14s0036g00520	L-allo-threonine aldolase	0.24156	0.114893	***0.623973***	0.275893
	2	VIT_14s0036g00520	L-allo-threonine aldolase	0.24156	0.114893	***0.623973***	0.275893
	3	VIT_06s0009g03740	serine-glyoxylate aminotransferase	***1.65717***	***0.789528***	0.0707028	0.319804
	4	VIT_06s0009g03740	serine-glyoxylate aminotransferase	***1.65717***	***0.789528***	0.0707028	0.319804
	4	VIT_00s0225g00130	Alanine transaminase.	***0.646075***	***0.325***	0.051118	0.0699332
Pathway B: Lys, Met & Thr metabolism	5	VIT_03s0038g02730	aspartate kinase	0.112512	-0.207648	0.569707	-0.0482074
	5	VIT_18s0001g03660	aspartate kinase	0.13123	0.0148823	0.00538204	-0.0678828
	5	VIT_01s0244g00140	aspartate kinase	0.167992	0.199927	0.244211	0.29053
	6	VIT_01s0011g05860	aspartate-semialdehyde dehydrogenase	0.0328198	0.262502	-0.090037	-0.259916
	7	VIT_15s0048g00750	dihydrodipicolinate synthase	-0.159871	0.0458089	***-0.373573***	***-0.453387***
	8	VIT_16s0100g00480	dihydrodipicolinate reductase	0.429921	-0.177307	-0.104241	-0.417615
	8	VIT_05s0094g01480	dihydrodipicolinate reductase	***0.31442***	0.145049	0.227692	-0.14041
	9	VIT_03s0088g01040	LL-diaminopimelate aminotransferase	0.0419909	-0.0512993	-0.088505	-0.187937
	9	VIT_12s0055g00920	LL-diaminopimelate aminotransferase	0	0	***-1.91744***	***-2.39183***
	9	VIT_03s0088g01060	LL-diaminopimelate aminotransferase	-0.0745544	-0.17	0.105797	-0.0070982
	9	VIT_18s0001g04630	LL-diaminopimelate aminotransferase	0.00951988	-0.180501	0.111843	-0.272836
	10	VIT_08s0040g02540	diaminopimelate epimerase	0.0330301	0.0504883	0.0926928	-0.141847
	11	VIT_09s0070g00580	diaminopimelate decarboxylase	-0.125401	-0.168998	-0.217699	0.000526875
	11	VIT_03s0038g04490	diaminopimelate decarboxylase	0.0371745	-0.0352219	0.0664809	-0.197634
	12	VIT_11s0016g00280	bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase	-0.0615955	-0.125547	-0.261125	-0.106693
	13	VIT_06s0009g01460	Homoserine dehydrogenase	0.137931	-0.0720496	0.22738	-0.225165
	14	VIT_11s0037g00340	homoserine kinase	-0.00397856	0.138798	-0.149099	-0.35157
	15	VIT_14s0108g01110	Cystathionine gamma-synthase isoform 2	***0.506823***	***0.415383***	***0.319926***	0.0950921
	15	VIT_08s0007g05410	Cystathionine beta-lyase	-0.0898196	0.0133722	0.150495	-0.266756
	15	VIT_19s0015g00380	Cystathionine beta-synthase	-0.105745	-0.0554111	-0.0342387	0.176187
	16	VIT_06s0009g01080	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	-0.205181	0.0703356	-0.13187	0.153564
	16	VIT_08s0056g01570	5-methyltetrahydropteroyltriglutamate--homocysteine S-methyltransferase	0.0312088	0.173035	-0.0258018	-0.22182
	17	VIT_07s0151g00520	homocysteine S-methyltransferase 3	0.0569109	0.220686	-0.0649772	0.00805911
	17	VIT_05s0020g03860	homocysteine S-methyltransferase 3	***1.40004***	0.23851	***0.793311***	-0.119619
	17	VIT_01s0011g03500	homocysteine S-methyltransferase 1	-0.433219	0.856105	0.0098787	2.00801
	18	VIT_15s0046g03700	3-beta hydroxysteroid dehydrogenase	-0.0539526	-0.0619725	-0.0264974	-0.115206
	18	VIT_14s0060g00480	S-adenosylmethionine synthetase 1 (SAM1)	0.125888	-0.0255345	-0.168015	***-0.403055***
	18	VIT_07s0005g02230	S-adenosylmethionine synthetase 1 (SAM1)	-0.254033	0.0770913	-0.216423	***-0.831424***
	18	VIT_05s0020g00670	S-adenosylmethionine synthetase 1 (SAM1)	0.276684	0.23935	0.0347419	-0.234455
	18	VIT_08s0007g05000	S-adenosylmethionine synthetase	-0.374717	***1.52473***	0.17232	***0.981448***
	19	VIT_11s0016g01720	threonine synthase	0.16207	0.133133	-0.291347	0.0485915
	20	VIT_14s0036g00520	L-allo-threonine aldolase	0.24156	0.114893	***0.623973***	0.275893
Pathway C: Trp, Phe & Tyr metabolism	21	VIT_06s0004g06320	anthranilate synthase component I-1, chloroplast precursor	-0.00966208	0.0646289	0.225322	0.0405366
	21	VIT_01s0011g06260	anthranilate synthase beta subunit	***0.329807***	0.269879	0.0804955	-0.27752
	21	VIT_13s0067g00400	anthranilate synthase alpha 1	***-0.646196***	***-0.833153***	-0.232996	-0.169768
	22	VIT_17s0000g08660	anthranilate phosphoribosyltransferase, chloroplast precursor	0.183011	0.0740361	0.130922	-0.246075
	22	VIT_14s0066g00970	anthranilate phosphoribosyltransferase, chloroplast precursor	0.113642	0.0903839	0.180127	0.127333
	22	VIT_00s0225g00150	anthranilate phosphoribosyltransferase	0.14397	-0.114979	0.217248	-0.0883105
	22	VIT_13s0067g01110	N-(5phosphoribosyl)anthranilate isomerase (PRAI)	0.0824349	0.235288	0.226143	-0.172934
	22	VIT_12s0057g01240	indole-3-glycerol phosphate synthase	0.11664	0.0271694	0.132119	0.126641
	22	VIT_07s0141g00210	tryptophan synthase, alpha subunit	0.00603479	-0.0748962	-0.0880505	-0.208319
	22	VIT_14s0083g00460	tryptophan synthase beta chain 2	-0.501453	1.35579	2.53202	***-1.91032***
	22	VIT_19s0177g00310	tryptophan synthase beta chain 1	0.00553336	-0.232146	-0.0663993	***0.481556***
	23	VIT_06s0009g03120	CYP79A2	-0.626363	***2.55569***	0.0493762	***-0.79239***
	24	VIT_14s0108g01330	chorismate mutase, chloroplast (CM1)	-0.139471	0.0204999	0.0393577	-0.159287
	24	VIT_04s0008g06570	chorismate mutase, cytosolic (CM2)	-0.0302313	0.371801	0.0298859	-0.0728089
	25	VIT_06s0061g01300	prephenate dehydratase	-0.280924	0.0963819	0.331886	-0.169744
	25	VIT_12s0059g00750	prephenate dehydratase	-0.0707537	0.351407	-0.315036	-0.203382
	25	VIT_06s0004g03430	prephenate dehydratase	-0.0478493	-0.00852188	-0.0392779	0.197742
	25	VIT_10s0116g01670	Prephenate dehydratase with ACT region	0.107062	-0.165465	0.673602	-0.28011
	26	VIT_06s0004g02620	PAL	***0.550934***	***0.760919***	***1.3141***	***1.26935***
	26	VIT_08s0040g01710	PAL	-0.036508	0.0413483	***0.640177***	***-1.2816***
	26	VIT_13s0019g04460	PAL	***0.378768***	0.126257	***0.683742***	***0.838955***
	27	VIT_13s0067g02120	arogenate dehydrogenase	-0.138477	0.268927	-0.0600844	-0.0761739
	27	VIT_09s0002g08070	prephenate dehydrogenase	-0.069092	-0.0578935	-0.184841	0.175269
	28	VIT_00s0225g00230	Tyrosine aminotransferase	0.492476	***1.34212***	***1.1095***	***-0.7839***
	28	VIT_00s0394g00040	Tyrosine aminotransferase	-0.872971	-0.353327	***1.25448***	***-1.20465***
Pathway D: Pro, Arg & GABA metabolism	29	VIT_08s0007g05600	pyrroline-5-carboxylate reductase	0.144652	0.0517682	0.157847	-0.00601223
	30	VIT_13s0019g02360	Pyrroline-5-carboxylate synthetase	0.143948	0.275412	0.184606	0.0389864
	31	VIT_14s0083g00520	proline oxidase	0.157797	0.170302	***1.10586***	***0.704886***
	32	VIT_17s0000g05800	delta-1-pyrroline-5-carboxylate dehydrogenase (P5CDH)	-0.127319	***-0.39607***	-0.143532	-0.131223
	33	VIT_04s0079g00600	Glutamate decarboxylase 1	0.168465	0.047716	-0.217853	***0.33661***
	33	VIT_01s0011g06610	Glutamate decarboxylase	-0.113712	-0.0217036	-0.347527	-0.520107
	33	VIT_01s0011g06600	glutamate decarboxylase	-0.262138	-0.350899	***-1.00014***	***0.729329***
	34	VIT_03s0038g00760	Arginine decarboxylase (Fragment)	0.096691	***0.438812***	-0.248346	0.113839
	35	VIT_15s0048g00420	Arginase 1	0.0752256	-0.0747143	0.0368401	-0.049336
	36	VIT_10s0003g03870	Ornithine aminotransferase	-0.262091	***-0.407322***	-0.150372	-0.291659
Pathway E: Ile, Val & Leu metabolism	37	VIT_08s0007g04310	Threonine dehydratase/deaminase	0.115679	-0.0379037	0.0786694	0.143147
	38	VIT_17s0000g01320	acetohydroxyacid synthase 3	-0.763966	-0.32277	-0.389432	-0.0456436
	38	VIT_05s0094g01110	acetolactate synthase small subunit	0.239833	0.0670689	0.154932	-0.0403764
	38	VIT_00s0220g00110	acetolactate synthase small subunit	-0.259888	0.154931	0.0731312	0.780545
	38	VIT_14s0068g01960	acetolactate synthase III, chloroplast precursor	***0.293297***	0.192175	-0.100429	0.0434197
	39	VIT_10s0003g01180	ketol-acid reductoisomerase	-0.764207	-0.834648	-0.470865	0.652564
	39	VIT_10s0003g01190	ketol-acid reductoisomerase	0.376609	0.0949737	-2.05919	1.05393
	39	VIT_12s0028g02340	ketol-acid reductoisomerase	0.2993	0.149189	0.119999	0.00656399
	39	VIT_05s0051g00830	dihydroxy-acid dehydratase	0.0168711	-0.203897	-0.138955	0.109249
	40	VIT_14s0128g00100	aminotransferase class IV	0.0763136	0.11515	0.0767844	-0.281232
	41	VIT_17s0000g01320	acetohydroxyacid synthase 3	-0.763966	-0.32277	-0.389432	-0.0456436
	41	VIT_05s0094g01110	acetolactate synthase small subunit	0.239833	0.0670689	0.154932	-0.0403764
	41	VIT_00s0220g00110	acetolactate synthase small subunit	-0.259888	0.154931	0.0731312	0.780545
	41	VIT_14s0068g01960	acetolactate synthase III, chloroplast precursor	***0.293297***	0.192175	-0.100429	0.0434197
	42	VIT_10s0003g01180	ketol-acid reductoisomerase	-0.764207	-0.834648	-0.470865	0.652564
	42	VIT_10s0003g01190	ketol-acid reductoisomerase	0.376609	0.0949737	-2.05919	1.05393
	42	VIT_12s0028g02340	ketol-acid reductoisomerase	0.2993	0.149189	0.119999	0.00656399
	42	VIT_05s0051g00830	dihydroxy-acid dehydratase	0.0168711	-0.203897	-0.138955	0.109249
	44	VIT_05s0124g00400	2-isopropylmalate synthase B	0.291954	0.102837	0.0761755	0.201287
	45	VIT_08s0056g01640	3-isopropylmalate dehydratase large subunit 2	0.216919	0.0516555	0.0740227	-0.00651487
	45	VIT_00s0551g00010	3-isopropylmalate dehydratase, small subunit	0.187649	0.247574	-0.230156	-0.261366
	45	VIT_05s0049g01980	3-isopropylmalate dehydratase large subunit 2	0.0579788	-0.0288288	***-0.369845***	-0.163087

Table S10: The metabolite concentrations and gene expression levels as indicated by the numbers assigned in Figure 8.

			Log2 Fold change (Exposed vs Control)			
Number in figure 8	Gene accession	Gene annotation (X12)	EL31	EL33	EL35	EL38
1	VIT_14s0030g00660	shikimate dehydrogenase	-0.51	-0.40	-0.51	0.25
1	VIT_14s0030g00650	shikimate dehydrogenase	0.23	0.09	0.27	***0.40***
2	VIT_18s0001g06250	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase	-0.23	0.07	-0.15	-0.19
2	VIT_00s1217g00010	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase	***0.43***	***0.34***	***0.30***	-0.10
2	VIT_00s0391g00070	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase	-0.14	0.22	-0.12	0.17
2	VIT_04s0023g03820	3-dehydroquinate synthase	-0.17	0.16	***-0.30***	-0.21
2	VIT_14s0030g00670	dehydroquinate dehydratase	-0.31	-0.48	0.01	-0.23
2	VIT_05s0020g02030	shikimate dehydrogenase	-0.04	0.07	-0.18	-0.26
2	VIT_14s0030g00660	shikimate dehydrogenase	-0.51	-0.40	-0.51	0.25
2	VIT_14s0030g00650	shikimate dehydrogenase	0.23	0.09	0.27	***0.40***
2	VIT_18s0001g01730	Shikimate kinase	-0.19	-0.51	0.01	-0.36
2	VIT_07s0031g01600	shikimate kinase	0.25	0.14	***0.35***	0.13
2	VIT_12s0142g00530	gamma-glutamyltranspeptidase	inf	-inf	-inf	0.00
2	VIT_15s0048g00350	3-phosphoshikimate 1-carboxyvinyltransferase, chloroplast precursor	0.11	0.17	0.03	0.03
2	VIT_13s0019g04190	Chorismate synthase 1, chloroplast precursor	0.06	0.10	-0.02	0.06
2	VIT_06s0004g02960	chorismate synthase 1	1.78	0.27	0.00	-inf
3	VIT_01s0011g06260	anthranilate synthase beta subunit	***0.33***	0.27	0.08	-0.28
3	VIT_13s0067g00400	anthranilate synthase alpha 1	***-0.65***	*-0.83*	-0.23	-0.17
3	VIT_14s0083g00460	tryptophan synthase beta chain 2	-0.50	1.36	2.53	***-1.91***
3	VIT_19s0177g00310	tryptophan synthase beta chain 1	0.01	-0.23	-0.07	***0.48***
4	VIT_06s0009g03120	CYP79A2	-0.63	***2.56***	0.05	***-0.79***
5	VIT_14s0108g01330	chorismate mutase, chloroplast (CM1)	-0.14	0.02	0.04	-0.16
5	VIT_04s0008g06570	chorismate mutase, cytosolic (CM2)	-0.03	0.37	0.03	-0.07
6	VIT_04s0008g06040	aspartate aminotransferase, chloroplast precursor	0.26	0.11	0.04	-0.14
6	VIT_18s0001g04860	Aspartate transaminase.	0.09	-0.10	-0.05	-0.19
6	VIT_07s0031g00980	aspartate aminotransferase	-0.06	0.15	0.09	***-0.50***
7	VIT_13s0067g02120	arogenate dehydrogenase	-0.14	0.27	-0.06	-0.08
7	VIT_09s0002g08030	arogenate dehydrogenase isoform 2	0.21	0.00	0.00	0.00
8	VIT_00s0394g00040	Alliinase EGF	-0.87	-0.35	***1.25***	***-1.20***
8	VIT_00s0225g00230	alliin lyase precursor	0.49	***1.34***	***1.11***	***-0.78***
9	VIT_10s0116g01660	lactoylglutathione lyase	***0.91***	***0.73***	***1.29***	***0.43***
9	VIT_11s0016g03440	lactoylglutathione lyase	0.19	0.01	-0.09	0.07
9	VIT_12s0028g00710	4-hydroxyphenylpyruvate dioxygenase	0.13	***0.64***	***-0.51***	***-0.53***
9	VIT_11s0052g00610	Homogenitisate geranylgeranyl transferase	-0.23	0.11	***-0.34***	-0.37
10	VIT_00s0179g00300	MPBQ/MSBQ methyltransferase 2	0.22	0.29	0.11	-0.25
10	VIT_16s0039g01410	tocopherol O-methyltransferase, chloroplast precursor	***0.43***	***0.39***	0.10	0.13
11	VIT_16s0039g01410	tocopherol O-methyltransferase, chloroplast precursor	***0.43***	***0.39***	0.10	0.13
12	VIT_07s0005g04480	tyrosine decarboxylase	0.12	***0.47***	***0.61***	***0.61***
13	VIT_13s0019g04540	GCN5 N-acetyltransferase (GNAT)	***0.33***	***0.83***	0.13	-0.07
14	VIT_10s0116g01670	Prephenate dehydratase with ACT region	0.11	-0.17	0.67	-0.28
14	VIT_06s0004g03430	prephenate dehydratase	-0.05	-0.01	-0.04	0.20
14	VIT_06s0061g01300	prephenate dehydratase	-0.28	0.10	0.33	-0.17
15	VIT_13s0019g04460	phenylalanine ammonia-lyase 2 (PAL2)	***0.38***	0.13	***0.68***	***0.84***
15	VIT_08s0040g01710	Phenylalanine ammonia-lyase	-0.04	0.04	***0.64***	***-1.28***
15	VIT_06s0004g02620	Phenylalanine ammonia-lyase	***0.55***	***0.76***	***1.31***	***1.27***
16	VIT_06s0004g08150	trans-cinnamate 4-monooxygenase	***0.36***	0.29	***0.44***	***0.68***
16	VIT_11s0065g00350	trans-cinnamate 4-monooxygenase	-0.41	0.41	-0.37	***0.84***
17	*VIT_08s0040g00780*	P-coumaroyl shikimate 3'-hydroxylase isoform 1	0.22	***0.56***	***0.85***	***0.64***
17	*VIT_11s0037g00440*	hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase	0.47	0.66	0.25	0.39
17	VIT_02s0025g03660	4-coumarate-CoA ligase	-0.02	0.11	-0.33	0.24
17	VIT_02s0109g00250	4-coumarate-CoA ligase	0.04	0.26	0.58	***0.68***
17	VIT_11s0052g01090	4-coumarate-CoA ligase 2	-0.23	-0.37	0.13	-0.34
17	VIT_16s0039g02040	4-coumarate-CoA ligase 3	***0.64***	***0.61***	***1.18***	0.26
17	VIT_16s0050g00390	4-coumarate-CoA ligase	***0.42***	***0.84***	-0.20	0.10
17	VIT_18s0001g00290	OPCL1 (OPC-8:0 COA LIGASE1)	-0.21	-0.10	-0.05	-0.17
17	VIT_02s0025g02920	quercetin 3-O-methyltransferase 1	0.49	***0.82***	***0.47***	0.18
18	VIT_08s0007g04520	caffeic acid 3-O-methyltransferase	0.00	0.14	-0.11	-0.21
18	VIT_16s0098g00850	Caffeic acid O-methyltransferase	0.12	***0.53***	0.01	-0.23
19	VIT_03s0063g00140	Caffeoyl-CoA O-methyltransferase	***-0.68***	0.06	0.47	-0.15
19	VIT_07s0031g00350	caffeoyl-CoA O-methyltransferase 1	-0.24	***1.09***	***0.91***	***0.61***
20	VIT_14s0068g00930	chalcone synthase 1	***0.59***	***0.92***	***0.48***	***1.07***
20	VIT_14s0068g00920	chalcone synthase 2	***0.81***	***1.20***	***1.52***	***1.96***
20	VIT_05s0136g00260	chalcone synthase 3	0.14	0.04	0.06	***-0.42***
21	VIT_13s0067g03820	chalcone isomerase [Vitis vinifera]	***0.39***	***0.59***	***0.41***	0.33
21	VIT_13s0067g02870	Chalcone-flavanone isomerase	0.09	0.15	***0.36***	0.07
22	VIT_18s0001g12800	Vitis vinifera dihydroflavonol reductase (DFR) mRNA XM_002281822.1	0.18	0.12	0.03	-0.15
23	VIT_04s0023g03370	flavanone-3-hydroxylase (F3H)	***0.47***	-0.16	0.25	***0.51***
23	VIT_18s0001g14310	Flavanone 3-hydroxylase (F3H)	***0.37***	***0.69***	***0.73***	***0.70***
24	VIT_18s0001g12800	Vitis vinifera dihydroflavonol reductase (DFR) mRNA XM_002281822.1	0.18	0.12	0.03	-0.15
25	VIT_18s0001g12800	Vitis vinifera dihydroflavonol reductase (DFR) mRNA XM_002281822.1	0.18	0.12	0.03	-0.15
26	VIT_02s0025g04720	leucoanthocyanidin dioxygenase (LDOX or ANS)	***0.39***	***0.37***	0.07	-0.32
26	VIT_08s0105g00380	Leucoanthocyanidin dioxygenase	***-0.52***	0.19	***-0.37***	-0.19
27	VIT_00s0361g00040	anthocyanidin reductase (BAN) [Vitis vinifera] GeneID: 100232981	0.17	***-0.58***	-0.24	0.41
28	VIT_18s0001g03430	Flavonol synthase	***-0.55***	***-0.76***	-0.27	***0.55***
28	VIT_18s0001g03470	Flavonol synthase Vitis vinifera	***4.73***	***3.53***	***3.85***	***3.75***
29	VIT_15s0046g00170	MybPA1	0.24	***0.57***	***0.94***	***1.23***
30	VIT_11s0016g01320	MybPA2	***0.43***	***0.78***	-0.05	inf

Chapter 4

The identification and evaluation of developmental and light responsive molecular biomarkers in Sauvignon Blanc grapes.

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The identification and evaluation of developmental and light responsive molecular biomarkers in Sauvignon Blanc grapes.

4.1 Introduction

The term "biomarker" was adopted in the medical field to describe a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention, as stipulated by the National Institute of Health (Strimbu and Tavel, 2011). Similarly, in plant research, a biomarker can act as a representative of a specific developmental stage in plant development or a specific response to a biotic or abiotic stress. A biomarker can therefore be a transcript, metabolite, molecule or protein. For example, developmentally driven biomarkers include a thaumatin-like gene that was identified as a marker associated with ripening and sugar accumulation of pepper plants (Kim et al., 2002) and a MAD-box gene was identified as a key biomarker in tomato ripening (Vrebalov et al., 2002). In the study of plant stress responses, Sadder et al., (2014) identified the molecular biomarkers responsive to drought, heat and salinity stress in tomatoes, while other biomarkers are frequently targeted to evaluate the toxicity of soil contaminants in several plant species (Singh and Prasad, 2014; Assad et al., 2017; Hou et al., 2015).

Because grapevine is the most important fruit crop worldwide, the development and ripening of grape berries have been studied at length. Grape berry development is known to follow a double sigmoidal curve. This curve involves the first stage of the development of berries when they are green, acidic and growing in size, whereas the ripening period is the developmental stage during which the sugars begin to accumulate, the berry softens and several aroma and phenolic compounds accumulate in order to attract seed dispersers (attractive smell and color) (Coombe et al., 1992; Coombe and McCarthy, 2000). These two developmental phases are separated by a transitional phase known as véraison (Coombe et al., 1992; Coombe and McCarthy, 2000). Véraison is further characterized by an oxidative burst that leads to the accumulation of non-lethal doses of reactive oxygen species (ROS) that are considered to be biomarkers for the onset of berry ripening (Pilati et al., 2007, 2014). Additionally, sugars and organic acids are well-described metabolic biomarkers of berry growth and development (Coombe, 1992; Topalovic & Mikulic-Petkovsek, 2010).

With the advent of high throughput transcriptomic analyses after the construction of the grapevine reference genome (Jaillon et al., 2007; Velasco et al., 2007), several subsequent investigations explored the gene expression underlying these two distinct developmental phases. Among these studies, a comprehensive

grapevine gene expression atlas established a clear distinction between global gene expression patterns in green, vegetative tissues and mature and/or woody tissues, confirming that there are several transcriptional switches that occur in berry development (Fasoli et al., 2012). Two high-throughput transcriptome studies further adopted the term "molecular biomarkers" in order to describe specific genes (or molecules) that correlate strongly with the physiological changes that take place during grape berry development (Zamboni et al., 2010; Palumbo et al., 2014).

Several biomarkers have also been identified for various stress conditions. For example, hexadecanoic acid was identified as a resistance marker to downy mildew (Batovska et al., 2009), whereas malondialdehyde acts as a biomarker for lipid peroxidation as a result of oxidative stress (Mittler et al., 2002). More recently, Liang et al. (2014) put forward a list of abiotic stress related molecular markers that the authors referred to as "Module 17". Module 17 was reported to contain 29 non-redundant genes in the grapevine genome that responded similarly to various abiotic stresses when the authors compared all public transcriptomic data available at the time. These studies provide a foundation of information with which to compare gene expression at specific grape developmental stages to determine whether the development of the berries under investigation are progressing according to the expected profile. Furthermore, the expression of a predetermined set of genes that characterize each grape developmental stage could hold valuable advantages in future investigation into grape berry metabolism. By identifying these predetermined developmentally driven gene expression profiles, researchers might be able to more accurately distinguish developmental patterns from those induced by a specific treatment or stress condition.

It was previously established that although the grapes evaluated in this investigation implemented several photoprotective mechanisms to mitigate the effects of elevated light exposure in the berry bunch zone, less than 10% of the berry transcriptome was affected by the treatment at any of the four developmental stages explored. These findings were further mirrored in the results pertaining to the physical and metabolic characteristics of the grapes. The grapes followed the well-established progression of development and the grapes exposed to elevated light were not physically different from those grown under shaded conditions when evaluated for berry weight or diameter, as well as sugar and acid contents and profiles (Young et al., 2016; Addendum B of Chapter 3). These findings point towards a highly coordinated molecular mechanism by which grape development is conserved regardless of impending abiotic stress. It would therefore by highly informative to identify the developmental stage-specific molecular biomarkers that represented the coordinated transition between green and ripening grapes and to ascertain which of these genes can be considered light stress markers in developing grapes.

Utilizing the concept of biomarkers, the aim was to identify two sets of biomarkers in order to fulfill two separate objectives. The first was aimed towards identifying developmental stage-specific molecular biomarkers

that are strongly correlated with the developmental pattern associated with grape berry development in our data and comparing these identified biomarkers to those previously identified (Zamboni et al., 2010; Palumbo et al., 2014. The identification of these biomarkers could potentially contribute to the refinement of a subset of genes with which to confirm the normal development of grapes under various growing conditions. These findings may further aid in distinguishing developmental from treatment-specific expression patterns in grapevine research.

The second objective was towards identifying, within the original set of markers identified, biomarkers that were significantly affected by elevated light exposure. These biomarkers would therefore be strongly associated with grape berry development under normal conditions, but would prove sensitive to light stress and could therefore potentially be considered as light stress biomarkers in future studies.

4.2 Materials and Methods

All methods pertaining to the experimental layout, growing conditions of the grapes, sampling strategy, RNA extractions and RNASeq have been described in previous sections of this thesis (Du Plessis et al. 2017; Young et al., 2016; Chapter 3). The putative developmental biomarkers were identified and further explored.

Firstly, the putative biomarkers that represent the transcriptional difference between the green and the ripening grape berry stages were identified according to the analysis pipeline established by Zamboni et al., (2010) with some modifications. In the aforementioned publication, the authors considered the green (EL33) and véraison (EL35) stages as early developmental stages compared to grapes from later developmental stages (EL36 and EL38). According to the RNASeq results generated and the initial characterization of the berry transcriptomes in this study, the transcriptional patterns of the grapes sampled at véraison (EL35) showed a higher Pearson correlation coefficient to later stage berries (EL38) than to green berries (EL31 and EL33) (Figure 1; Chapter 3). Therefore, as the first modification to the established analysis pipeline, in this evaluation, grapes sampled at EL31 and EL33 was categorized as “early” and EL35 and EL38 were categorized as “late” stages. A two-class OPLS-DA model was generated by representing the expression of green, control berry samples (EL31 and EL33) as its own class as a reference against expression of ripening, control berry samples (EL35 and EL38) set as the second class using SIMCA (version 14.0). An S-plot was subsequently generated to identify the loading correlation coefficient of each gene as described by Zamboni et al. (2010; Wiklund et al., 2008).

The second modification made to the established protocol pertained to the correlation cut-off parameters implemented. The aim of this investigation was to initially generate a broad overview of the developmental progression of the grapes included in this study and therefore, a less stringent correlation cut-off was implemented than in previous studies to identify genes with a loading correlation coefficient higher than 0.8 (positive biomarkers) and lower than −0.8 (negative biomarkers). Hereby, positive biomarkers would

theoretically represent genes that are expressed at higher levels during the late stages (EL35 and EL38) compared to early stages (EL31 and EL33) and will therefore be referred to as "late markers". Conversely, negative biomarkers would represent genes that show higher expression during early stages than late stages and will subsequently be referred to as "early markers". Line graphs representing biomarker expression across the four stages of berry development was generated in the Multi-experiment Viewer (MeV; Saeed et al., 2006).

To explore the functional characteristics of these biomarkers, Gene Ontology (GO) Enrichment analyses were performed of the early and late developmental biomarkers separately in the BiNGO application in Cytoscape (version 3.4.0) using the Benjamini and Hochberg False Discovery Rate Correction metric. GO terms were considered significant with a *p*-value smaller than 0.05. By targeting the results generated from differential expression analysis, the developmental biomarkers that were significantly affected by elevated light exposure could be identified. Those that were not significantly different when comparing the FPKM expression values of the exposed and control grapes will be referred to as "unaffected".

In order to establish whether these identified control grape berry developmental biomarkers were comparable to those already established for grape developmental progression, molecular biomarkers identified in this investigation were compared to those published from two previous investigations. The first set of biomarkers included in this comparison was published by Zamboni et al. (2010) in which transcriptional elements unique to early berry development (EL33 and EL35) and late berry development (EL36 and EL38) were identified and named Class a and Class b genes, respectively (adopted from the terminology used in Zamboni et al., 2010). These biomarkers will be referred to as early and late developmental markers in subsequent sections of this chapter.

The second set of genes used to compare the development of the grapes included in this study was published by Palumbo et al. (2014) in which the authors identified so-called "switch genes" that are considered to characterize the unique transcriptional switch that occurs when grape berries transition from being green, photosynthesizing organs to becoming ripening, sink organs. This aforementioned study utilized transcriptional data generated from five red Italian grape cultivars as well as data generated from the grapevine transcription atlas (Fasoli et al., 2012). A Venn diagram was constructed using the Bioinformatics and Evolutionary Genomics platform (http://bioinformatics.psb.ugent.be/webtools/Venn/) by comparing the genes from the abovementioned studies and the molecular biomarkers identified in this study.

Using the identified developmental biomarkers, the effect of the light treatment on the expression of these biomarkers could be established. This was achieved by determining which of the identified biomarkers shared between this and previous studies were significantly affected by elevated light.

4.3 Results and Discussion

4.3.1 Identification and characterization of functional associations of the putative developmental stage-specific biomarkers in developing Sauvignon Blanc grapes.

In total, 4975 genes were identified as developmental stage-specific biomarkers responsible for the greatest transcriptional differences between the green and ripening developmental stages. There were 2734 and 2241 early and late developmental markers that showed a correlation coefficient greater than 0.8 (80%), respectively. The expression of early biomarkers was significantly higher during the early developmental stages (EL31 and EL33) than in the later, ripening stages (EL35 and EL38) and the late biomarkers showing the opposite expression pattern (Figure 4.1).

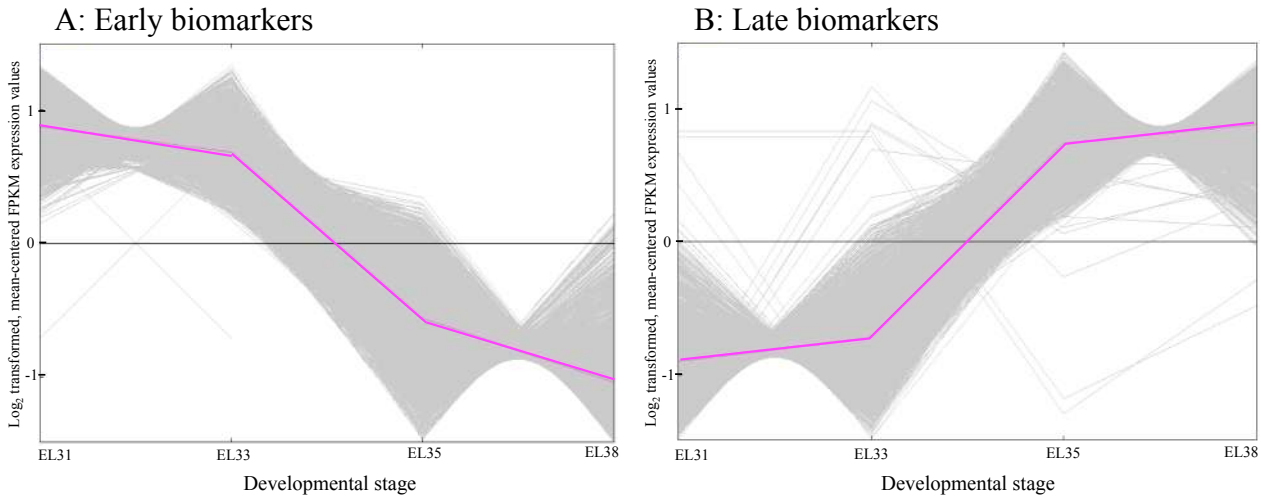

Figure 4.1. The expression patterns of the developmental biomarkers identified when comparing early development (EL31 and EL33) and late development (EL35 and EL38) of Sauvignon Blanc grapes. A: Log_2 transformed, mean centered expression levels (FPKM-values) of the 2734 early developmental biomarkers at four phenological stage of berry development. B: Log_2-transformed, mean-centered expression levels (FPKM-values) of the 2241 late developmental biomarkers at four phenological stage of berry development.

GO Enrichment analysis of these biomarkers revealed that the early developmental markers were significantly enriched (p<0.05) for functions associated with photosynthesis (Figure 4.2). This functional annotation represented 37 genes that encode several integral thylakoid membrane proteins associated with the light harvesting complexes of photosystem I (PSI) and photosystem (PSII) as well as several chloroplast precursor proteins, hereby ensuring effective photosynthesis and biomass accumulation when the berries are still actively growing in size. Additionally, 19 of these genes are similarly represented by the GO term “generation of precursor metabolites and energy”.

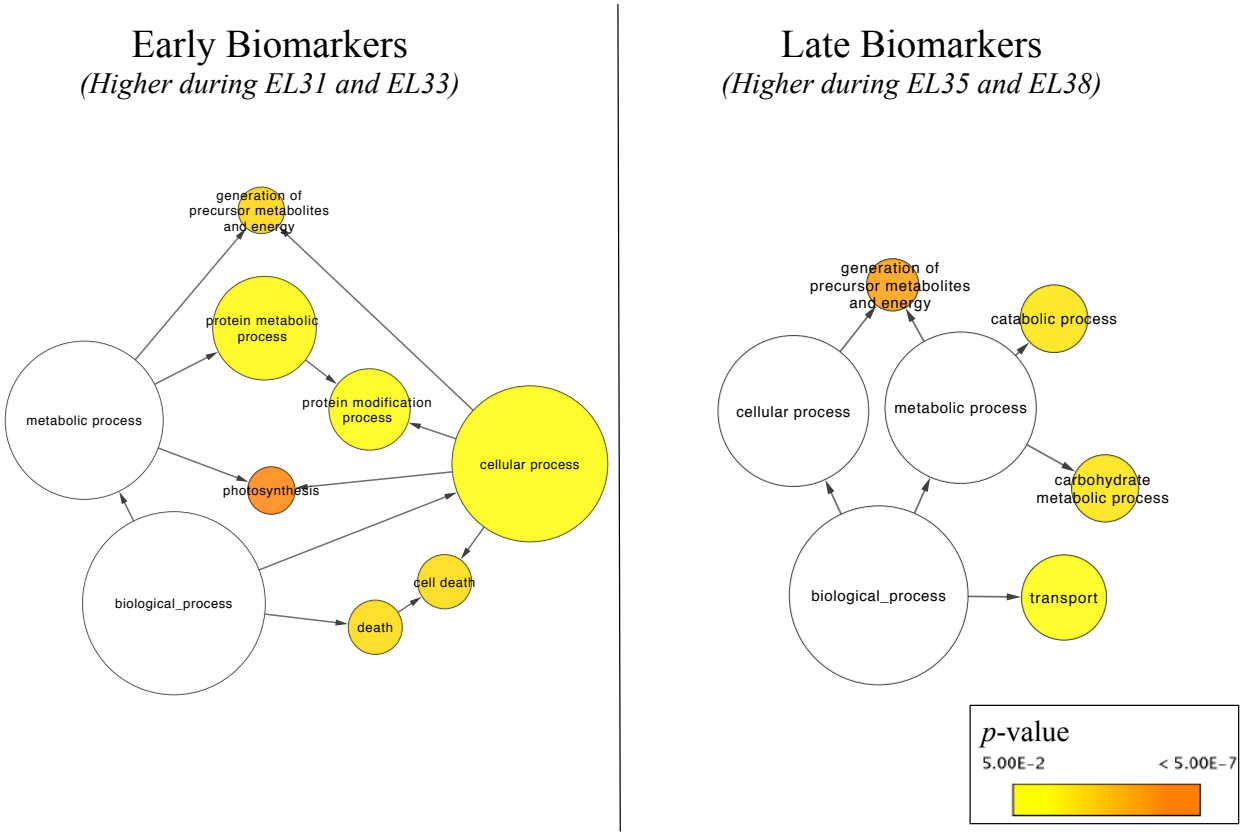

Figure 4.2. GO Enrichment analysis of the molecular biomarkers identified when comparing early (EL31 and EL33) to late (EL35 and EL38) developmental stages in developing grapes grown under control conditions. Colored nodes indicate significantly enriched GO terms (p<0.05).

The GO annotations, "death" and "cell death" was further significantly enriched among the early biomarkers and represented 52 genes almost exclusively associated with disease resistance. It was, however, previously established that these grapes did not experience any notable disease pressure (Young et al., 2016). These findings therefore possibly point towards the mechanisms associated with disease resistance during the early berry developmental stages during which the grape seed is still developing, contributing to the ontogenic resistance of green grapes to various pathogens such as *Botrytis cinerea* (reviewed in Elmer and Reglinski, 2006) and *Erysiphe necator* that causes powdery mildew (Gadoury et al., 2003).

The late biomarkers were functionally enriched for GO terms associated with the generation of precursor metabolites and energy, catabolic processes, carbohydrate metabolism and transport (Figure 4.2). The genes represented by the first three abovementioned GO terms are, however, shared between these functions. For example, 22 out of the 33 genes represented by the GO term "generation of precursor metabolites and energy"

are also represented by the GO term "catabolic process" of which 18 genes are also shared with the term "carbohydrate metabolic process". These genes are predominantly involved in the process of glycolysis and the synthesis of sugars such as glucose and hexose associated with the accumulation of sugars during the process of berry ripening.

Interestingly, although the GO term "generation of precursors metabolites and energy" is similarly significantly enriched in the early and late biomarkers, the genes represented by these functional annotations are distinctly different when comparing early and late markers. During the early developmental stages, this GO term represents genes associated with photosynthesis whereas during the ripening stages this term represents genes associated with sugar accumulation.

Taken together, these results point towards the well established, highly coordinated progression of grape berry development on a transcriptional level (extensively reviewed in Serrano et al., 2017) and are further supported by results presented earlier (Addendum B; Young et al., 2016; Chapter 3). According to these earlier findings, the development of the Sauvignon Blanc grapes in this study progressed as expected, with sugar accumulating gradually and organic acid concentrations decreasing as ripening progressed, as supported by the enrichment of GO annotations associated with energy, precursor and carbohydrate metabolism during later stages. Although the dynamics of photosynthesis differ when comparing foliar and non-foliar tissues, green fruits, such as grapes, are photosynthetically active, (Blanke and Lenz, 1989; Kyzeridou et al., 2015), hereby supporting the strong association with the photosynthetic machinery among the early developmental biomarkers.

4.3.2 The identification and establishment of grape berry developmental stage-specific molecular biomarkers consistently driven by developmental cues, regardless of treatment or genotype.

The identification and establishment of the validity of developmental stage-specific biomarkers were performed in three steps. Firstly, all of the developmental biomarkers identified in the preceding section (2734 early and 2241 late biomarkers) were compared to previously published developmental markers identified in grapevine in order to identify the genes that were similarly identified. These will be referred to as "shared biomarkers". Secondly, these shared biomarkers were further evaluated to determine whether they are affected by elevated light. Those that responded consistent throughout development, regardless of light exposure, were referred to as "unaffected". Finally, the unaffected shared biomarkers were targeted in the transcriptomes generated from the grapes of ten Italian cultivars throughout development and compared to the Sauvignon Blanc transcriptomes generated in this study and is presented in Figure B4.1; Addendum B of Chapter 4.

4.3.2.1 Comparison of developmental stage-specific biomarkers to previously published markers

The exploration of the molecular biomarkers identified in this study revealed that the expression of these markers were comparable to previously established markers for grape berry development. More than half of the "switch genes" identified by Palumbo et al. (2014) and 28 of the biomarkers identified in Zamboni et al. (2010) were also identified as either early or late developmental biomarkers in this study (Figure 4.3). Interestingly, none of the switch genes (Palumbo et al., 2014) were similarly identified as either class a (early) or class b (late) biomarkers according to Zamboni et al. (2010). This may be attributed to the fact that Zamboni et al. (2010) included the transcriptomes of EL35 berries to the early stages of development, whereas Palumbo et al. (2014) included EL35 berry transcriptomes to the late stages of development (similar to our study). This difference in experimental layout is further evident by the fact that class a markers (EL33 and EL35) published by Zamboni et al., (2010) showed overlap with both early and late biomarkers identified in this study. Among these shared early markers is a chloroplast precursor protein encoding gene (VIT_03s0180g00130) whereas the one gene shared between the class a (early markers) and the late biomarkers identified in this study encoded a thioredoxin protein (VIT_08s0007g07620). Thioredoxins are responsible for scavenging reactive oxygen species (ROS) generated under abiotic stress conditions in chloroplasts in order to sustain photosynthetic activity in *Arabidopsis* (Serrato et al. 2004). An oxidative burst that coincides with accumulation of non-lethal levels of ROS however signifies the onset of berry ripening at véraison (Pilati et al., 2007, 2014) that could explain why this thioredoxin encoding gene is associated with this developmental stage. These findings are further supported by the fact that Terrier et al. (2016) similarly reported the expression of a thioredoxin gene as a biomarker for grape berry ripening in a cultivar-dependent manner.

Among the late biomarkers shared with the switch genes (Palumbo et al., 2014) was a gene involved in the degradation of carotenoids (9-cis-epoxycarotenoid dioxygenase) associated with the enzymatic breakdown of the photosynthetic light-harvesting carotenoids, typically synthesized during the early developmental stages (Zhang et al., 2009; Young et al., 2012). Additionally, two of these late markers were ferritin encoding genes that have been implicated in the reduction of oxidative stress induced by the transition towards ripening in strawberries (Aharoni et al., 2002). All of the biomarkers depicted in the Venn diagram (Figure 4.3) and their functional annotations are listed in Table A4.1; Addendum A of Chapter 4.

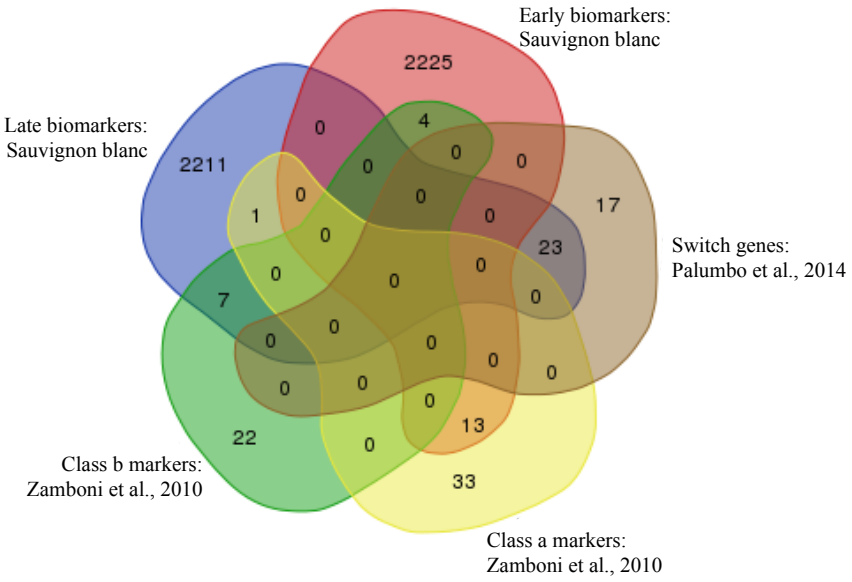

Figure 4.3. Venn diagram comparing the molecular biomarkers generated in this study (Sauvignon blanc) to previously published biomarkers from Zamboni et al., (2010) and Palumbo et al., (2014).

4.3.2.2 Identification of shared developmental biomarkers that remain unaffected by elevated light exposure

In order to further explore the validity in considering these genes as developmental biomarkers representative of grape berry development, their expression patterns were explored. All of the shared biomarkers (both early and late) were targeted in differential expression analysis comparing the expression of these genes in light exposed and control (shaded) grapes. The aim of this analysis was to determine whether these genes were purely involved in the developmental progression of the grapes or whether their expression could be altered by abiotic conditions. Out of the 51 biomarker genes shared between this investigation and previous studies, eleven biomarkers were not significantly affected (q>0.05) by light (Figure 4.4). Five and six of these genes represented early and late developmental markers, respectively. According to these results, eleven biomarkers are not affected by the abiotic treatment applied in this study and may therefore be indicative of genes that drive berry development, regardless of the treatment.

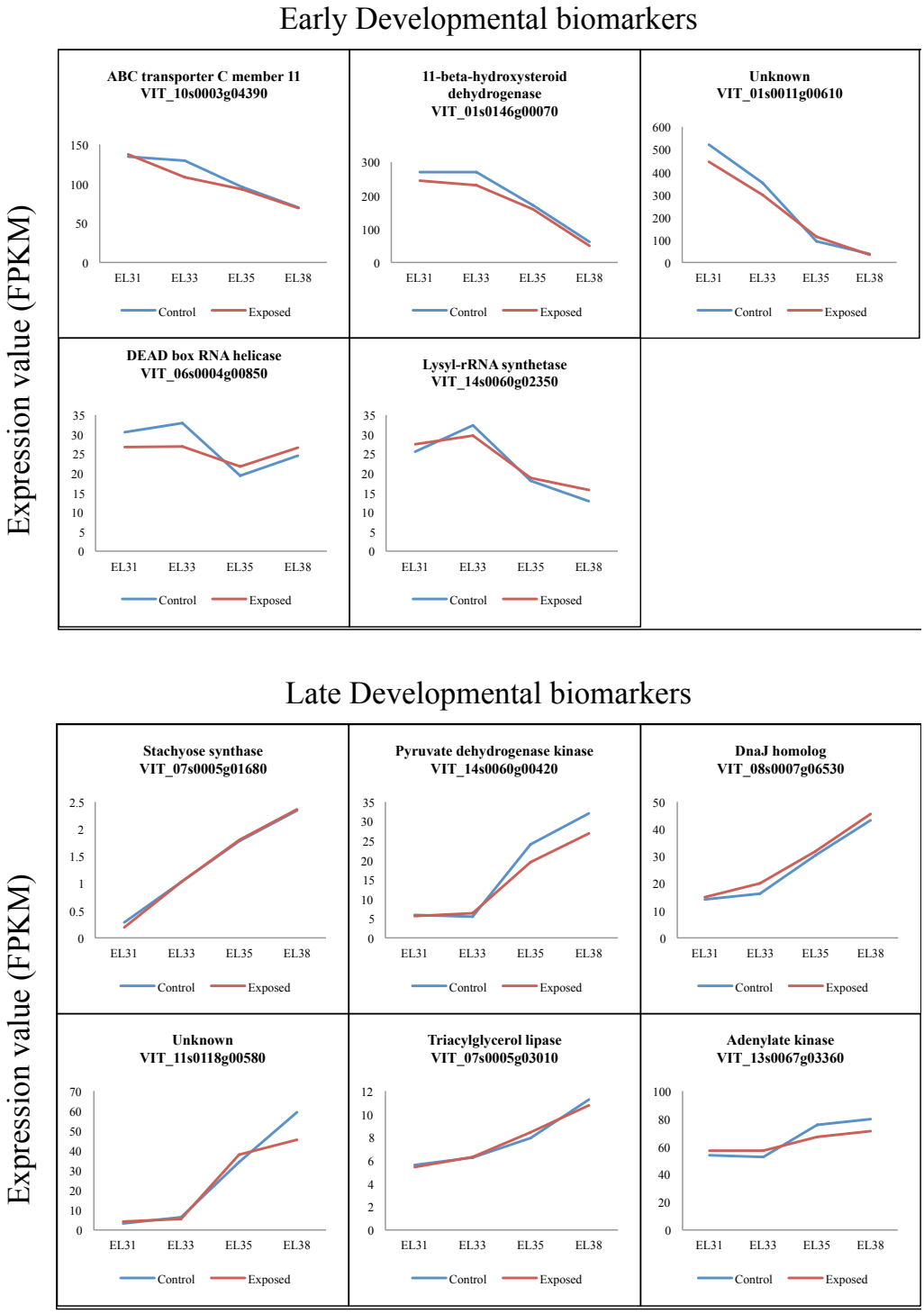

Figure 4.4. Line graphs representing the average expression level (FPKM) of the eleven biomarkers shared between this study and those previously reported that are not significantly affected by elevated exposure (q>0.05) in the berry microclimate. Low variability in expression levels (FPKM) between biological replicates has been established previously (Chapter 3).

Among the early developmental markers that remained unaffected by light, was a DEAD-box RNA helicase gene (VIT_06s0004g00850). In *Arabidopsis*, DEAD-box RNA helicases were found to be responsible for the attenuation of several abiotic stresses through epigenetic silencing of gene expression (Kant et al., 2007; Kahn et al., 2014). Furthermore, the 11-beta-hydroxysteroid dehydrogenase gene was unaffected by light treatment and was also previously reported as a biomarker associated with grape berry development (Terrier et al., 2016).

One of the late developmental biomarkers that were unaffected by light exposure was a stachyose synthase encoding gene (VIT_07s0005g01680) that was previously implicated in grape fruit ripening and softening processes (Nicolas et al., 2013). Similarly, Wang et al., (2017) reported the upregulation of a pyruvate dehydrogenase kinase gene in a developmentally driven manner after véraison.

In an attempt to further establish these genes as grape berry developmental biomarkers, the expression of these genes were analyzed in the RNASeq data generated form the transcriptomes of ten Italian cultivars (Figure B4.1; Addendum B of Chapter 4). The results generated from a Hierarchical Clustering analysis revealed two main conclusions. Firstly, out of the eleven biomarkers analyzed, eight have similar expression, with only three showing a slightly dissimilar expression in five cultivars sampled at véraison. These genes included a triacylglycerol lipase (VIT_07s0005g03010), adenylate kinase (VIT_13s0067g03360) and a DnaJ homolog (VIT_08s0007g06530).

The second conclusion confirmed our rationale behind grouping EL35 (véraison) with the other ripening samples, instead of with the earlier developmental stages before véraison. The expression of these eleven biomarkers clearly separated green from ripening samples, showing conclusively that véraison grapes are transcriptionally more similar to ripe grapes in these ten grapevine cultivars when comparing the developmental biomarkers identified in Sauvignon Blanc. Interestingly, véraison samples from five cultivars clustered uniquely from the other ripening samples. Four of these samples represented white grape cultivars.

Taken together, according to the criteria applied, the developmental stage-specific biomarkers identified in this investigation are potential candidates to represent the molecular mechanisms associated with transition between early development when the berries are green to the later ripening stages of berry development, regardless of the level of light these grapes are exposed to. Although these results provide preliminary insights into the expression patterns of these biomarkers in ten other cultivars, future studies into their expression in various other cultivars could further refined this list of proposed molecular biomarkers associated with berry development and ripening. These biomarkers did, however, show developmental stage-specific expression in Sauvignon Blanc grapes, comparable to previous reports (Zamboni et al., 2010; Palumbo et al., 2014) and were not affected by elevated light exposure.

4.3.3 Identification of potential light-stress biomarkers that are significantly affected by light exposure throughout grape berry development.

Utilizing the results generated from the differential expression analysis comparing control to light exposed grapes, the putative developmental stage specific biomarkers that were significantly affected (q<0.05) by elevated light could be identified. More than half of the biomarkers remained unaffected by elevated light. Specifically, 1488 (54%) of early markers and 1318 (59%) of late markers did not show significant differential expression during the developmental phases in response to exposure. The biomarkers that were significantly affected by exposure at both of the developmental phases were further explored.

The early biomarkers that showed consistent significantly affected expression during all four of the developmental stages evaluated are listed in Table 4.1. Among the most significantly upregulated genes were predominantly heat shock proteins (Hsps) such as Hsp81, Hsp101, several sHsps and a Heat shock factor (*VviHsfA6b*) putatively responsible for their regulation (Hong and Vierling, 2000; Huang et al., 2016). These Hsps are known to be involved in the implementation of abiotic stress mitigation mechanisms, however, they are also implicated as molecular chaperones involved the efficient functioning of the turnover of thylakoid membrane proteins under normal developmental conditions as reported and extensively reviewed recently (Guo et al., 2016; Jacob et al., 2017; Neta-Sharir, 2005; Nishizawa-Yokoi et al., 2011; Park et al., 2015; Scharf et al., 2012; Zhong et al., 2013). This may explain why these Hsps are early biomarkers under non-stressed conditions but that they become significantly upregulated in response to elevated light exposure in order to protect the plant from protein damage throughout berry development through their chaperone activity (reviewed in Jacob et al., 2017). These findings are further supported by the fact that several of these consistently affected genes encode proteins of the PSI and PSII during the early developmental stages as well (Table 4.1).

These findings are further corroborated by the results presented in the previous chapter (Chapter 3; Du Plessis et al., 2017). Photosynthesis and the genes encoding proteins of the photosynthetic machinery were the most significantly altered in response to the light exposure treatment during the early, green stages of development. However, the primary metabolic processes were not affected on a transcriptional or metabolic level (Du Plessis et al., 2017; Young et al., 2016). These findings collectively point towards the turnover of the photosynthetic machinery through the upregulation of Hsp-encoding genes.

Table 4.1. A table representing the early developmental biomarkers and their functional annotations that were significantly affected by elevated light exposure throughout berry development. The expression patterns of the biomarkers are summarized from A to D, indicating the log_2 transformed, mean-centered expression values (FPKM) of each of the genes. The values indicated in the table is the log_2 fold change when comparing exposed to control grapes at each developmental stages. All of these comparisons were significantly different (q<0.05).

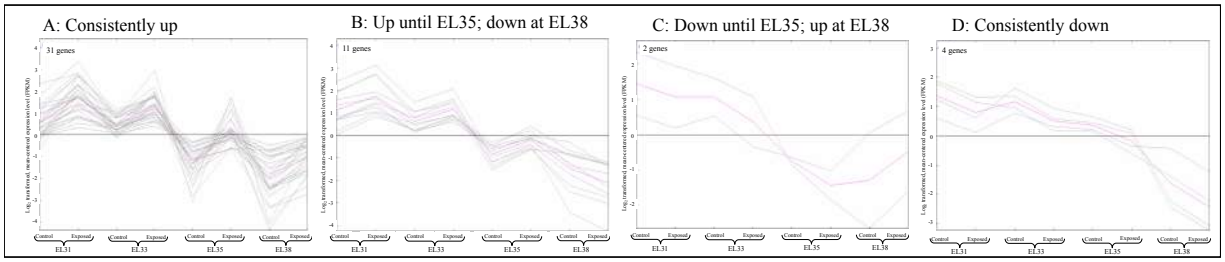

Diagram	Gene annotation (12X)	Gene accession	Log_2 Fold Change (Exposed vs Control)			
			EL31	EL33	EL35	EL38
A	Heat shock protein 81-1	*VIT_02s0025g00280*	2.99	2.13	4.01	2.02
	Heat shock protein 16.9 kDa class I	*VIT_13s0019g02740*	2.98	2.21	4.82	3.19
	Heat shock transcription factor A6B	*VIT_00s0179g00150*	2.26	1.71	3.27	2.81
	Heat shock protein 101	*VIT_17s0000g07190*	2.10	1.48	2.98	1.54
	Chaperonin	*VIT_01s0011g04990*	1.97	1.51	2.76	0.98
	1-aminocyclopropane-1-carboxylate oxidase homolog 1	*VIT_05s0049g00300*	1.70	0.81	1.40	1.26
	E3 ubiquitin-protein ligase COP1	*VIT_16s0050g00020*	1.66	1.44	1.87	1.53
	No hit	*VIT_05s0102g00620*	1.60	0.56	2.08	0.59
	Unfertilized embryo sac 10 UNE10	*VIT_15s0021g02690*	1.57	0.96	2.24	1.78
	Cyclopropane fatty acid synthase	*VIT_05s0049g01650*	1.55	1.34	2.72	2.57
	Heat-shock protein 18.6 kDa	*VIT_13s0019g03170*	1.54	1.28	2.36	0.73
	LHB1B1	*VIT_12s0028g00320*	1.52	1.36	1.29	0.82
	CGA1 (CYTOKININ-RESPONSIVE GATA FACTOR 1)	*VIT_04s0008g01290*	1.52	1.04	0.96	2.25
	Short-chain dehydrogenase/reductase	*VIT_16s0050g00750*	1.32	1.03	1.48	1.01
	Fructose-bisphosphate aldolase, chloroplast precursor	*VIT_04s0023g03010*	1.27	0.72	0.48	0.54
	Unknown protein	*VIT_02s0025g02640*	1.22	0.86	0.64	1.14
	Quinone oxidoreductase, chloroplast precursor	*VIT_01s0127g00740*	1.15	0.87	1.42	0.84
	Dehydrogenase	*VIT_12s0059g01150*	0.91	0.54	0.51	0.43
	heat shock protein 83	*VIT_12s0057g00670*	0.71	0.54	0.99	0.53
	Ubiquitin-conjugating enzyme E2 D/E	*VIT_06s0004g08200*	0.59	0.56	0.49	0.41
	Steroleosin-B	*VIT_18s0157g00200*	0.55	0.34	0.73	0.42
	CBS domain-containing protein	*VIT_00s0587g00030*	0.43	0.52	1.19	0.51
	Leucoanthocyanidin reductase 2 [Vitis vinifera] GENE ID: 100	*VIT_17s0000g04150*	0.42	0.49	0.52	0.96
	Glucan endo-1,3-beta-glucosidase 5 precursor	*VIT_00s0780g00020*	0.40	0.35	0.46	0.67
	CCR4-NOT transcription complex protein	*VIT_09s0070g00060*	0.39	0.34	0.50	0.39
	Thioredoxin family	*VIT_07s0005g06420*	0.74	0.65	0.67	0.53
	DnaJ homolog, subfamily B, member 6	*VIT_09s0002g00690*	0.88	0.53	1.12	0.57
	Anthranilate N-benzoyltransferase protein 1	*VIT_03s0038g01330*	0.80	0.81	1.05	1.26
	WD-40 repeat	*VIT_18s0001g05220*	0.72	0.72	0.65	0.32
	Mitochondrial substrate carrier family protein	*VIT_05s0020g00480*	0.83	0.63	2.52	1.25
	Mg-chelatase subunit XANTHA-F	*VIT_08s0007g08540*	0.82	0.41	0.53	0.72
B	Photosystem I subunit XI (PSAL)	*VIT_04s0023g00410*	0.84	0.76	0.78	-0.52
	Nodulin 1A, Senescence-associated	*VIT_03s0063g01150*	0.82	0.58	0.45	-1.11
	Chaperonin 10, Chloroplast	*VIT_15s0021g00830*	0.81	0.59	0.84	-0.53
	Photosystem II oxygen-evolving enhancer protein PSBQ	*VIT_00s0904g00010*	0.77	0.58	0.61	-0.54
	No hit	*VIT_16s0098g00600*	0.76	0.48	0.92	-0.47
	Photosystem I reaction center subunit N (PSAN)	*VIT_04s0044g01410*	0.73	0.60	0.60	-0.84
	Magnesium-protoporphyrin O-methyltransferase	*VIT_17s0000g00280*	0.71	0.56	0.45	-0.41
	DegP protease,	*VIT_18s0072g00970*	0.41	0.39	0.41	-0.45
	Heavy-metal-associated domain-containing protein / copper ch	*VIT_06s0061g00440*	0.35	0.36	0.50	-0.37
	Triacylglycerol lipase	*VIT_12s0028g02000*	0.34	0.34	0.77	-0.89
	GRAM domain-containing protein / ABA-responsive	*VIT_17s0119g00110*	0.31	0.49	0.63	-1.04
C	Phytochrome-associated protein 1 (PAP1)	*VIT_09s0002g03410*	-0.36	-0.88	-0.39	0.61
	Unknown protein	*VIT_14s0006g00920*	-0.40	-0.53	-0.99	1.05
D	Amidase	*VIT_15s0024g00980*	-0.49	-0.44	-0.56	-0.80
	TIR-NBS-LRR-TIR disease resistance protein	*VIT_18s0117g00050*	-0.55	-0.75	-0.35	-0.81
	Unknown protein	*VIT_08s0007g01490*	-0.63	-0.80	-0.77	-0.85
	CYP72A1	*VIT_15s0021g01040*	-0.64	-0.69	-0.45	-0.85

The late biomarkers that were consistently significantly affected by elevated light throughout berry development are reported in Table 4.2. These biomarkers include a flavanone 3-hydroxylase (F3'H) encoding gene (VIT_18s0001g14310) and one of the transcription factors that regulate its expression, MYB domain protein (VIT_18s0001g09850) in grapevine post véraison (Matus et al., 2009). The F3'H enzyme is involved in flavonoid biosynthesis that has been previously shown to be among the compounds accumulated at higher levels in response to elevated light in these grapes (Du Plessis et al., 2017; Chapter 3). Therefore, these findings draw a strong transcriptional link between the differentially expressed late biomarkers and the accumulation of higher concentration flavonoid compounds with photoprotective qualities.

Two other interesting biomarker candidates emerged from these results. The first was a galactinol synthase encoding gene (VIT_01s0127g00470). This enzyme is involved in the first enzymatic step in the synthesis of first galactinol and then provides galactosyl towards the synthesis of raffinose and stachyose. In grapevine tissues, galactinol synthase encoding genes have been reported to be upregulated in response to heat stress (Lecourieux et al., 2017; Pastore et al., 2017; Pillet et al., 2012; Rienth et al., 2016), high light exposure (Carvalho et al., 2011), leaf removal treatment (Pastore et al., 2013), abscisic acid treatment (Nicolas et al., 2014) and water deficit (Cramer et al., 2007) in various grapevine tissues. These genes were further identified as part of Module 17 in which the authors compiled a list of grapevine molecular biomarkers that are consistently upregulated by various abiotic stress exposures. Interestingly, these module 17 markers have not been tested for their response to elevated light in grape berries, confirming it being a reliable indicator for general abiotic stress status in developing grapes, including exposure stress.

The second interesting candidate for a light-stress molecular biomarker is the early light inducible protein gene (ELIP; VIT_05s0020g04110). Under non-stressed conditions, ELIP proteins have been associated with the transition from chloroplasts in green tomatoes to chromoplasts in ripening tomatoes (Bruno and Wetzel, 2004), however, these proteins are more frequently studied in the context of light stress (reviewed in Li et al., 2009). Pinto et al., (2011) studied the ELIP proteins in grapevine leaves and confirmed their role in photoprotection by showing that their accumulation is directly proportional to the light intensity applied to the leaves. Other studies have further reported the involvement of ELIP proteins in grapevine leaves exposed to elevated light (Carvalho et al., 2011; Li et al., 2017; Nilo-poyanco et al., 2013).

In *Arabidopsis*, the suppression of ELIP synthesis caused excessive foliar bleaching and severe photooxidative damage (Hutin et al., 2003). These findings confirmed the photoprotective function of ELIPs and the authors proposed that these proteins are either involved in the turnover of the pigment-binding proteins under high light stress by binding the chlorophylls being released or by aiding in the assembly and/or stabilization of the newly formed proteins under these conditions (Hutin et al., 2003).

The results presented here further contribute by confirming that ELIP gene expression is significantly upregulated in response to elevated light exposure to developing Sauvignon Blanc grapes in which the turnover of the proteins involved in photosynthesis has been established (Chapter 3). Furthermore, the expression pattern of this gene remained consistent when comparing RNASeq data to results from Real-time PCR expression analysis of the same gene (Chapter 3), making this gene a potential candidate to represent light-stress biomarkers in developing grapes.

Future studies could contribute to these results by determining whether this ELIP encoding gene is affected by any other abiotic stresses in order to determine whether this gene could serve specifically as a light-stress biomarker or rather as a general abiotic stress marker.

Table 4.2. A table representing the late developmental biomarkers and their functional annotations that were significantly affected by elevated light exposure throughout berry development. The expression patterns of the biomarkers are summarized from A to D, indicating the log_2 transformed, mean-centered expression values (FPKM) of each of the genes. The values indicated in the table is the log_2 fold change when comparing exposed to control grapes at each developmental stages. All of these comparisons were significantly different (q<0.05).

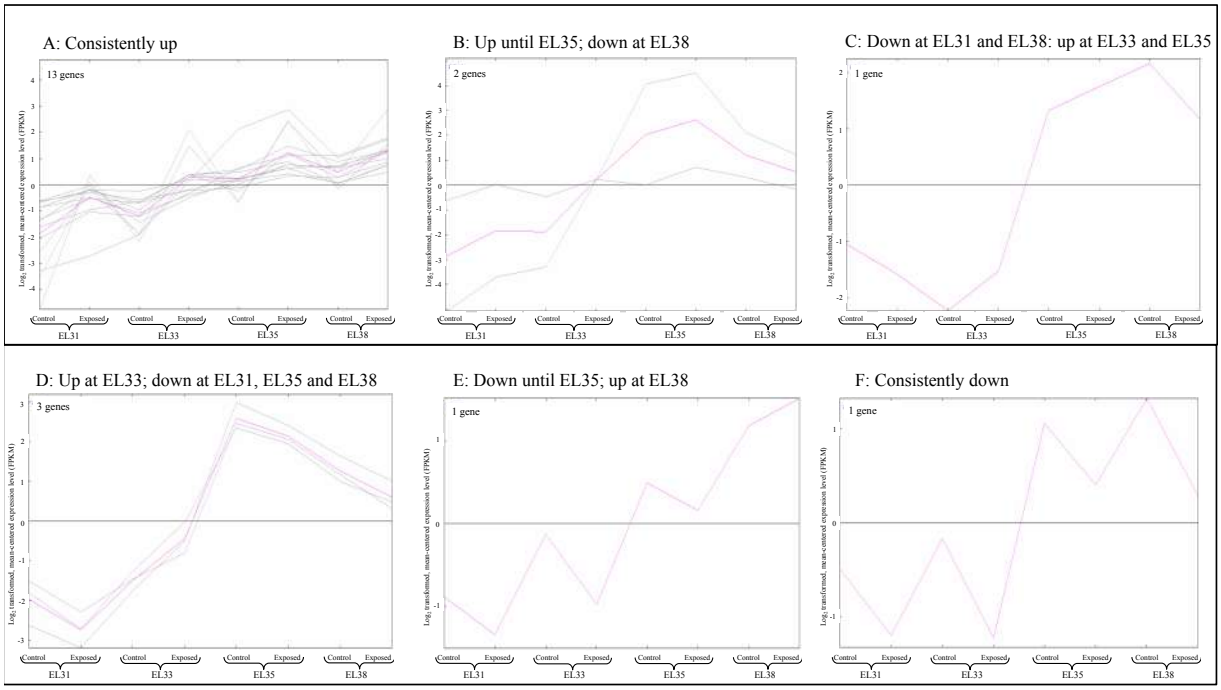

Diagram	Gene annotation (12X)	Gene accession	Log_2 Fold Change (Exposed vs Control)			
			EL31	EL33	EL35	EL38
A	Heat shock protein Cytosolic class II low molecular weight	*VIT_04s0008g01520*	5.12	3.64	3.08	2.39
	Unknown protein	*VIT_07s0005g01080*	3.62	3.99	3.06	1.70
	ELIP1 (Early Light-Inducible Protein)	*VIT_05s0020g04110*	2.36	2.27	0.91	0.42
	Rhomboid	*VIT_05s0020g01120*	1.20	1.34	1.43	0.55
	Dehydration-responsive element-binding protein 2C	*VIT_13s0067g01960*	1.02	0.88	0.47	0.65
	ATELC/ELC	*VIT_15s0021g02790*	0.80	1.08	0.40	0.66
	TIC55 (Translocon at the inner envelope membrane of chloroplasts 55)	*VIT_04s0008g07020*	0.67	0.44	0.29	0.40
	Molecular chaperone DnaJ	*VIT_19s0015g01370*	0.65	1.00	0.37	0.48
	Myb domain protein R1	*VIT_18s0001g09850*	0.58	0.94	0.60	0.35
	Galactinol synthase	*VIT_01s0127g00470*	0.56	2.02	0.76	0.70
	Unknown protein	*VIT_13s0067g02090*	0.47	0.47	0.46	1.02
	Flavanone 3-hydroxylase (F3H)	*VIT_18s0001g14310*	0.37	0.69	0.73	0.70
	ABC transporter I member 9	*VIT_14s0060g00720*	0.34	0.37	0.43	0.75
B	Gibberellin 20 oxidase 2	*VIT_03s0063g01290*	1.39	3.48	0.45	-0.90
	Protein kinase APK1B	*VIT_03s0038g03800*	0.67	0.70	0.71	-0.46
C	Unknown protein	*VIT_02s0025g01450*	-0.54	0.71	0.42	-1.00
D	Expansin [Vitis labrusca x Vitis vinifera] EXPA15	*VIT_01s0026g02620*	-0.58	1.25	-0.57	-0.63
	CTP-synthetase	*VIT_01s0010g00240*	-0.79	0.65	-0.40	-0.85
	Binding	*VIT_01s0026g01020*	-0.90	1.23	-0.40	-0.51
E	Pseudo-response regulator 5 (APRR5)	*VIT_16s0098g00900*	-0.45	-0.85	-0.33	0.33
F	Unknown	*VIT_05s0020g01080*	-0.70	-1.05	-0.66	-1.04

4.4 Conclusion

Here we report on the developmental stage-specific biomarkers that signify the greatest transcriptional differences between the green (EL31 and EL33) and ripening (EL35 and EL38) berry developmental stages. The findings reported here contribute to the refinement of the potential molecular biomarkers associated with the highly coordinated transcriptional progression associated with grape berry development, by confirming a small subset of previously established markers in Sauvignon blanc grapes. The developmental stage specific biomarkers put forward here not only showed overlap with previously established biomarkers in other studies, but was not affected by a elevated light or cultivar differences among those included in this study. Future studies could further refine this list of biomarkers by targeting these genes in various other grapevine genotypes and stress-related treatments. These biomarkers provided further insights into how these grapes maintained normal growth and development despite being exposed to elevated light. The establishment of a grapevine pan-genome could further contribute to the confirmation of specific genes that remain conserved in developing grapes of a wide range of grapevine cultivars.

Additionally, this analysis put forward a list of abiotic stress-related biomarkers that are developmentally regulated under non-stressed conditions, but that show significantly altered expression patterns in response to light stress. Some of these genes confirmed and enriched previous findings by adding light-stress in grape berries to the known abiotic stresses that these genes are markers for. Others may be novel candidates for the indication of light-stress in developing grape berries that were not reported previously. One of these candidates included an ELIP encoding gene that was shown to be involved in the turnover of proteins involved in photosynthesis in *Arabidopsis*, similar to findings reported earlier in this study. Future studies into the potential role of this light-sensitive biomarker may elucidate its role as a chaperone in grape berries responding to elevated light.

The study of stress-responsive molecular biomarkers may benefit from an updated analysis of the currently available transcriptomic data for grape berries to further elaborate on the current repertoire of known stress-responsive molecular biomarkers.

4.5 References

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Addendum A to Chapter 4

This Addendum contains relevant and additional data not shown in Chapter 4.

Table A4.1. Differential expression analysis of biomarkers shared between this investigation and previously published biomarkers as represented in Figure 4.3 (Chapter 4).

		Significantly different (q<0.05) between exposed and control					
Gene ID	Functional Annotation (12X)	EL31	EL33	EL34	EL38	Shared with	Unaffected
VIT_07s0005g01680	stachyose synthase					Switch	Always
VIT_14s0060g00420	pyruvate dehydrogenase kinase					Switch	Always
VIT_11s0118g00580	unknown					Switch	Always
VIT_07s0005g03010	triacylglycerol lipase					Class b	Always
VIT_13s0067g03360	adenylate kinase					Class b	Always
VIT_08s0007g06530	DnaJ homolog, subfamily B, member 6					Class b	Always
VIT_01s0011g00610	Unknown protein					Class a	Always
VIT_01s0146g00070	11-beta-hydroxysteroid dehydrogenase					Class a	Always
VIT_10s0003g04390	ABC transporter C member 11					Class a	Always
VIT_06s0004g00850	DEAD box RNA helicase					Class b	Always
VIT_14s0060g02350	lysyl-tRNA synthetase					Class b	Always
VIT_16s0100g00570	dehydration-responsive protein			***0.02***		Switch	Green stages
VIT_08s0040g01950	zinc finger (C3HC4-type RING finger)			***0.00***		Switch	Green stages
VIT_14s0066g01190	Unknown protein			***0.01***		Switch	Green stages
VIT_15s0048g02280	NAC transcription factor-like 9			***0.01***		Class a	Green stages
VIT_08s0058g00410	ferritin 1 (FER1)				***0.01***	Switch	Green stages
VIT_01s0150g00040	Unknown protein				***0.00***	Class b	Green stages
VIT_09s0002g00980	proteasome 20S alpha subunit A1				***0.02***	Class b	Green stages
VIT_12s0028g03380	ADP,ATP carrier protein				***0.02***	Class a	Green stages
VIT_03s0017g01210	Phosphate-induced protein 1				***0.00***	Class a	Green stages
VIT_16s0022g00670	Vacuolar invertase 1, GIN1				***0.01***	Class a	Green stages
VIT_03s0180g00130	D-3-phosphoglycerate dehydrogenase, chloroplast precursor				***0.00***	Class b	Green stages
VIT_06s0004g00620	ACCELERATED CELL DEATH 1 ACD1	***0.01***				Class a	Ripening stages
VIT_15s0046g02910	Ribosomal protein L21, chloroplast / CL21 (RPL21) 50S	***0.00***				Class a	Ripening stages
VIT_06s0004g03910	Unknown protein		***0.00***			Switch	Ripening stages
VIT_02s0087g00930	9-cis-epoxycarotenoid dioxygenase		***0.00***			Switch	Ripening stages
VIT_01s0127g00680	SRO2 (SIMILAR TO RCD ONE 2)		***0.00***			Switch	Ripening stages
VIT_19s0027g00230	NAC domain-containing protein 22		***0.00***			Switch	Ripening stages
VIT_04s0044g01230	no hit		***0.00***			Switch	Ripening stages

VIT_02s0025g04340	osmotin		***0.00***			Switch	Ripening stages
VIT_08s0040g01200	short-chain type alcohol dehydrogenase		***0.00***			Switch	Ripening stages
VIT_00s0181g00080	F-box domain containing protein		***0.00***			Class b	Ripening stages
VIT_08s0007g08840	Glycosyl transferaseHGA1	***0.01***	***0.00***			Switch	Ripening stages
VIT_16s0050g00390	4-coumarate-CoA ligase	***0.00***	***0.00***			Switch	Ripening stages
VIT_09s0002g06420	lactoylglutathione lyase	***0.01***	***0.00***			Switch	Ripening stages
VIT_14s0219g00040	zinc finger (C3HC4-type RING finger)	***0.02***	***0.00***			Switch	Ripening stages
VIT_08s0007g07620	thioredoxin 2	***0.00***	***0.00***			Class a	Ripening stages
VIT_18s0089g01390	Unknown protein	***0.00***	***0.01***			Class a	Ripening stages
VIT_14s0068g01950	Ribosomal protein L27, chloroplast (RPL27) 50S	***0.00***	***0.00***			Class a	Ripening stages
VIT_16s0098g01150	Auxin-responsive SAUR29		***0.00***	***0.00***		Switch	
VIT_14s0108g00450	no hit		***0.00***	***0.02***		Switch	
VIT_14s0006g01030	Calmodulin-binding heat-shock protein		***0.00***	***0.00***		Class b	
VIT_12s0028g01080	Photosystem II oxygen-evolving complex precursor, 23kda PSBP	***0.00***	***0.00***	***0.00***		Class a	
VIT_05s0020g03180	Photosystem I reaction center subunit II, chloroplast precursor	***0.00***			***0.00***	Class a	
VIT_08s0058g00440	ferritin		***0.00***		***0.01***	Switch	
VIT_14s0068g01760	Alcohol dehydrogenase		***0.00***	***0.01***	***0.00***	Switch	
VIT_00s0214g00090	F-box protein PP2-B10 (Protein PHLOEM PROTEIN 2-LIKE B10)		***0.00***	***0.00***	***0.00***	Switch	
VIT_16s0050g00750	short-chain dehydrogenase/reductase	***0.00***	***0.00***	***0.00***	***0.00***	Class b	

Addendum B to Chapter 4

This Addendum contains relevant and additional data not shown in Chapter 4.

B4.1 Materials and methods

In order to explore the expression patterns of the small subset of developmental stage-specific biomarkers in other cultivars, RNASeq data generated in this investigation was compared to the expression of these genes in 5 red and 5 white Italian cultivars as generated by RNASeq analysis (Massonnet et al., 2017). The expression values of these biomarkers were normalized separately by dividing each expression value with the average expression value calculated for all the biomarkers within each experiment, respectively. Hereby, the ratio of expression within each experiment could be effectively compared between different experiments by taking the inherent differences between the experimental methods and/or practices into account. The expression patterns between various the various cultivars were explore through Hierarchical clustering analysis in the Multi-Experiment Viewer (MeV; Saeed et al., 2006).

B4.2 Results

B4.2.1 Evaluating the effect of genotype specific expression patterns of the shared developmental biomarkers that are unaffected by light exposure

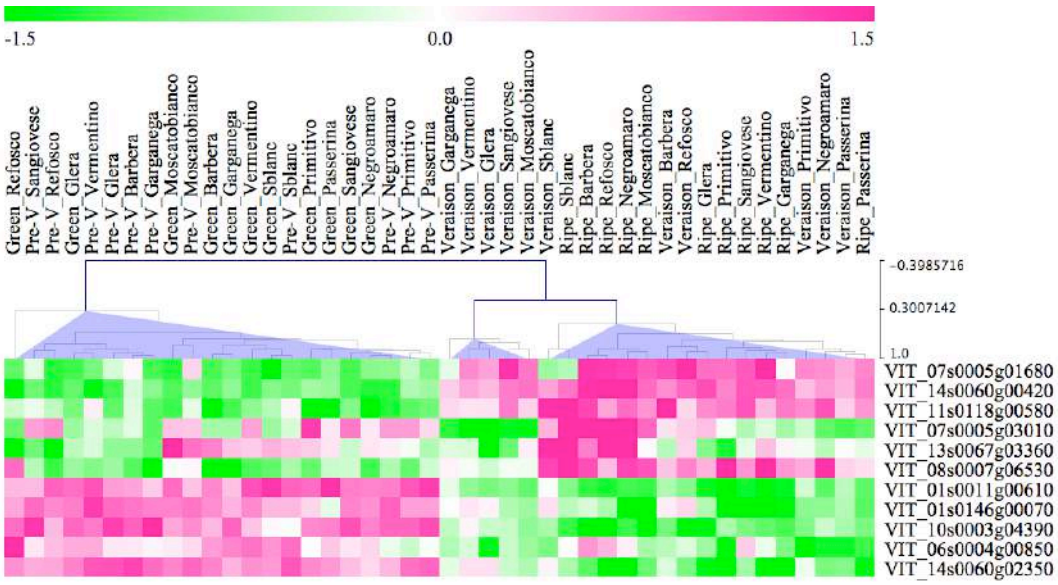

Figure B4.1. Hierarchical clustering analysis results depicting the relative* expression of each of the putative developmental biomarkers in eleven *V. vinifera* berry genotypes.* Refers to the expression ratio relative to each separate experiment (described in materials and methods).

B4.3 References

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Chapter 5

Heat shock factor, HsfA7a, may be involved in grape berry acclimation to elevated light exposure

Chapter 5

Heat shock factor, HsfA7a, may be involved in grape berry acclimation to elevated light exposure

5.1 Introduction

Heat shock proteins (Hsps) are ubiquitous proteins found in animal and plant cells. These proteins were initially identified by Ritossa (1962) in Drosophila exposed to heat stress and were subsequently described in model plant systems such as *Arabidopsis* responding to temperature stress as well (Lindquist & Craig, 1988). Although Hsps are important for plant cellular homeostasis and growth under normal, non-stressed conditions (Che, 2002; Giorno et al., 2010; Liu et al., 2011; Ikeda et al., 2011; Pick et al., 2012), these proteins have become synonymous with plant abiotic stress responses. Their expression and synthesis are known to be induced in response to a wide range of abiotic stresses that include heat and cold stress, light exposure and salinity stress during which they act as molecular chaperones responsible for the stabilization and refolding of misfolded proteins damaged as a result of the stress, as recently extensively reviewed by Jacob et al. (2017).

These Hsps form part of a superfamily of proteins that can be further classified into five Hsp protein sub-families characterized by the approximate molecular weights (kDa) of their members. These sub-families include Hsp100, Hsp90, Hsp70, Hsp60 and a family of small Hsps (sHsps) with a molecular weight lower than 50 (Vierling et al., 1991 for a review). The members of each of these sub-families perform unique and distinct roles in plant cellular homeostasis and are regulated by specific combinations of transcription factors known as Heat shock factors (Hsfs). Hsfs were first identified and grouped according to three known classes in *Arabidopsis*. The functional roles of these Hsfs are considered to be linked to their structures. Class a Hsfs are considered to act as transcriptional activators of Hsps-encoding genes, as well as a wide range of other genes, through their short activator peptide motifs (AHA motifs) located in their C-terminal domains. Contrastingly, class b Hsfs lack the AHA motif required for transcriptional activation and are considered to rather act as transcriptional repressors that are crucial for the attenuation of plant stress responses as part of stress recovery. Class c Hsfs also lack the activator domain and very few studies have focused toward their characterization and an understanding of their functions remains limited (reviewed in Yabuta, 2016).

Although the diversity and evolution of Hsfs in the plant kingdom has been explored and extensively reviewed, our current knowledge regarding the specific roles of Hsfs in plants remain limited at this time (reviewed in Scharf et al., 2012). Several targeted studies in *Arabidopsis* utilized Hsf knockout mutants to study the role that these Hsfs may play in normal growth and abiotic stress responses. These studies revealed tremendous functional diversity associated with the different Hsfs and that these proteins can frequently not

replace each other in order to fulfill their specific functions. For example, knock-out mutants of *HsfA1* in tomato revealed that none of the other Hsfs could replace its role as the master regulator in thermotolerance and the knockout mutants were not only impaired in their response to high temperatures, but showed growth abnormalities as well (Mishra et al., 2002).

More recently several studies were focused towards the genome-wide identification and expression analysis of *Hsfs* in various non-model (crop) plant systems that included potato (Tang et al., 2016), strawberry (Hu et al., 2015; Liao et al., 2016), banana (Wei et al., 2016), apple (Giorno et al., 2012), carrot (Huang et al., 2014), ponkan fruit (Lin et al., 2015), Chinese cabbage (Song et al., 2014) and the wild Chinese grapevine, *Vitis pseudoreticulata* (Hu et al., 2016). Collectively, these studies aimed to characterize the roles of Hsfs in abiotic stress resistance.

In grapevine, the upregulation of *Hsps* and their regulatory *Hsfs* are frequently reported in transcriptomic experiments performed under a wide range of abiotic stresses in vegetative tissues (Carvalho et al., 2015; Liu et al., 2012; Pontin et al., 2010; Rocheta et al., 2014; Wang et al., 2010) as well as in berries (Jiang et al., 2017; Pastore et al., 2013; Rienth et al., 2016; Sun et al., 2015), however, their involvement in the molecular mechanisms of abiotic stress responses have not been fully explored. Two studies reported targeted analysis of the expression patterns of *Hsps* in grapevine through real-time PCR (Banilas et al., 2012; Pillet et al., 2012). The first reported the expression profiles of the grapevine *Hsp90* family genes in grapevine leaves responding to heat shock treatments (Banilas et al., 2012). The authors confirmed that the expression characteristics of these *Hsps* were similar to their *Arabidopsis* orthologs, but that the expression patterns among the members of the *Hsp90* family were highly complex when responding to various intensities of temperature stress in the different grapevine tissues. They also reported that one of the *Hsp90* members was expressed in tendrils in the absence of stress (Banilas et al., 2012).

The second report targeted the expression of one *Hsf* (*HsfA2*) and its co-expression with a galactinol synthase encoding gene in greenhouse grown grape berries exposed to heat stress (Pillet et al., 2012). This study revealed that the known stress marker, galactinol synthase, is encoded for by the galactinol synthase encoding gene, *VviGOLS1*, that is transactivated by HsfA2 in a heat stress dependent manner. The authors proposed that galactinol synthase may be involved as a stress signaling molecule, since its downstream compounds, raffinose and stachyose, did not accumulate as a result of its upregulation (Pillet et al., 2012).

Furthermore, as part of the construction and validation of a co-expression network in grapevine, Liang et al., (2014) identified a cluster of grapevine genes that behaved as molecular stress-related biomarkers in response to a wide range of abiotic stresses. The authors identified this gene cluster, now referred to as "module 17", by utilizing all of the whole-transcriptome datasets publically available at the time in a co-expression network. Module 17 therefore represents 29 genes that are significantly upregulated by salt, water deficit and high temperature stress, as well as in micropropagated grapevine plantlets transferred to *ex*

vitro conditions. Among the 29 genes within module 17, 19 are *Hsps* from either the Hsp20, Hsp40, Hsp70, Hsp90 and Hsp100 families. The authors further confirmed the sensitivity of these module 17 genes to heat stress, but their expression responses to other abiotic (stress) factors have not been reported in any grapevine tissues.

The most recent study that focused on the expression of *Hsfs* in *V. pseudoreticulata* greatly contributed to our understanding of *Hsfs* in the *V. vinifera* genome. By using the 29 putative grapevine (*V. vinifera*) *Hsf* encoding genes described in the Plant Transcription Factor Database (Plant TFDB; Jin et al., 2015, 2017) the authors performed phylogenetic, synteny and redundancy analyses to ultimately identify nineteen non-redundant Hsf encoding genes in the *V. vinifera* genome. The authors identified these *V. vinifera* Hsfs as a means to evaluate the expression of these genes in their species of interest (Hu et al., 2016), however, the abiotic stress responses of these *Hsfs* were not further evaluated in *V. vinifera*.

Our study of the global transcriptomic response of Sauvignon Blanc grapes exposed to more light revealed that the expression of a substantial number of *Hsps* and *Hsfs* was highly and significantly altered (Du Plessis et al., 2017; Chapter 3). The RNASeq dataset generated in this study therefore provided an opportunity to study the expression patterns of the grapevine *Hsf* genes in developing grapes (also in the tissue-types of the grape berries and the other plant organs of grapevine, as well as in other cultivars) and to further evaluate how these genes respond to elevated light exposure. Here we provide an overview of the expression of *Hsfs* in *V. vinifera*, building on the knowledge regarding grapevine *Hsfs* generated by Hu et al., (2016) to ultimately develop a working model to understand how *Hsp* and *Hsfs* encoding genes were involved in the grape berry acclimation responses to increased exposure.

5.2 Materials and Methods

All methods pertaining to the experimental layout, growing conditions of the grapes, sampling strategy, RNA extractions and RNASeq have been described in previous sections of this thesis (Du Plessis et al. 2017; Young et al., 2016; Chapter 3).

In order to construct a complete Sauvignon Blanc transcriptome, a representative dataset was constructed by utilizing filtered fragment reads generated by the RNASeq analysis from whole-berry (control and exposed – reported in Chapters 3 and 4; as well as RNASeq analysis from skin and pulp samples from the control grapes). This data subset combined 330 million fragments suitable for a *de novo* assembly of the Sauvignon Blanc transcriptome that was subjected to digital normalization using the Khmer software package (Version 1.1; Crusoe et al., 2014). The total remaining 17 050 123 fragments were used to perform the *de novo* assembly with the Velvet/Oases pipeline (Version 0.2.08; Schulz et al., 2012). A detailed description of the method pipeline including the data used, the digital normalization, the *de novo* assembly, clustering, redundancy removal and selection of the final transcripts are included in Addendum A to Chapter 5.

5.2.1 The Hsf encoding genes in the grapevine genome and their expression patterns

In order to explore and characterize the expression of Hsf encoding genes in the grapevine genome (version V1), accessions of these genes were obtained from a recent study in which they were identified (Hu et al., 2016). In the present study, the NCBI accessions for each of the nineteen Hsf genes were extracted from the abovementioned publication and their nucleotide sequences obtained with which a nucleotide BLAST was performed in the Grape Genome Database of Cribi (Centro di Ricerca Interdipratimentale per le Biotecnologie Innovative; Vitulo et al., 2014) in order to identify the gene accessions (VIT_) and the currently available functional annotation for each putative *VviHsf*.

A nucleotide BLAST was performed for each of the *VviHsf* cDNA sequences generated by *de novo* transcriptome assembly for Sauvignon Blanc in Cribi (Vitulo et al., 2014) in order to identify the level of identity between these gene sequences represented in the Sauvignon Blanc transcriptome and Pinot noir reference genome (PN40024).

5.2.2 Analysis of light induced transcriptional responses

Hierarchical cluster analysis of *VviHsf* gene expression in developing grapes under control and exposed conditions was performed in Multi-Experiment Viewer (MeV; Saeed et al., 2006). The expression data was $\log_2$ transformed and mean centered after which a Pearson correlation metric was implemented to generate a sample and gene tree grouping both samples and genes based on similar expression profiles.

Co-expression analysis was performed to identify genes that were putatively co-expressed within the RNASeq dataset for the berry pericarp samples using the Comparative Co-Expression Network Construction and Visualization tool (CoExpNetViz; Tzfadia et al., 2016). This method of co-expression analysis explores the correlation between certain genes and not causality. For the identification of overlapping genes sets, Venn-diagrams were constructed using the Bioinformatics & Evolutionary Genomics platform tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) and drawn in Microsoft PowerPoint (version 14.1.0).

GO enrichment analyses were performed using the online analysis tool, AgriGO (Du et al., 2010) using the Fisher statistical method with the Yekutieli False Discovery Rate multitest adjustment metric. Significantly enriched GO terms (p<0.05) were further visualized and summarized using the Reduce + Visualize Gene Ontology Web Server (http://revigo.irb.hr; Supek et al., 2011).

For the purpose of comparing co-expressed genes with previously identified abiotic stress-related markers in grapevine, the genes identified within ‘Module 17’ (Liang et al., 2014) were utilized to contextualize the findings of this investigation. Module 17 was reported to contain 29 non-redundant genes in the grapevine

genome that responded similarly to various abiotic stresses when the authors compared all public transcriptomic data available at the time.

5.3 Results

The RNASeq dataset generated from whole Sauvignon Blanc berries utilized for this investigation has previously been characterized and verified for validity and repeatability and is available on the NCBI's GEO under the series accession, GSE98873 (Du Plessis et al., 2017). An overview of the transcriptional data generated is available in Chapter 3 of this thesis.

The *de novo* construction of the Sauvignon Blanc transcriptome yielded 21 083 transcripts that mapped uniquely to transcripts in the *V. vinifera* genome assembly (12X), with a further 4137 and 2993 transcripts that either mapped to multiple transcripts or were identified as putative chimeras, respectively. Putative chimeras were either transcripts that were partially mapped on different chromosomes or that align to different regions of the same chromosome. There were further 220 transcripts identified that did not map to any transcripts in the *V. vinifera* genome (12X) – these need further characterization, but could potentially be linked to Sauvignon Blanc specific features. The final statistics of the transcript constructions are presented in Table A5.2; Addendum A to Chapter 5.

5.3.1 Identification and classification of Heat Shock Factors in the grapevine genome

A total of nineteen genes encoding Hsfs were previously identified in the grapevine genome (Hu et al., 2016) and the accessions of these genes were targeted to identify the same genes in the RNASeq datasets generated in this study. In an attempt to account for the potential differences between the Pinot noir and the Sauvignon Blanc genomes, the 19 *VviHsfs* were targeted in the *de novo* assembled Sauvignon Blanc transcriptome. This allowed for the characterization of the level of identity (%) between the sequences of the nineteen *VviHsfs* represented in the Pinot noir genome and Sauvignon Blanc transcriptome. The gene accessions, their characteristics, the level of identity between the two cultivars and the currently available functional associations of these *Hsf* genes reported in other plant species are summarized in Table 5.1.

Two out of the nineteen *VviHsfs* could not be identified in the Sauvignon Blanc transcriptome and another three *Hsfs* were either found to align with multiple hits or were found to be putative chimeras in the Sauvignon Blanc transcriptome (Table 5.1). By performing a nucleotide BLAST analysis with each of the *VviHsf* sequences identified in the Sauvignon Blanc transcriptome, it was established that 14 of these genes were more than 99% identical when comparing these genes in the Pinot noir and Sauvignon Blanc transcriptomes.

Table 5.1. Details pertaining to the *VviHsfs* represented in the *de novo* transcriptome assembly of Sauvignon Blanc in comparison to the Pinot noir reference genome (PN40024).

Class	Gene name	Gene accession	Nucleotide length (bp)	Molecular Weight (kDa)	Mapping on *V. vinifera* genome	Sauvignon blanc identity (%)	Treatment associated Hsf expression (other plant systems)	References
A	*VviHsfA1a*	VIT_02s0012g01810	1977	54.38	uniquely mapping	99.85	Elevated temperature, Salt stress, osmotic tolerance	Liu and Charng, 2013
	VviHsfA1b	VIT_16s0039g01840	2180	56.84	uniquely mapping	99.70	Elevated temperature, Salt stress, osmotic tolerance, oxidative stress	Liu and Charng, 2013
	VviHsfA2a	VIT_04s0008g01110	1556	44.16	uniquely mapping	99.88	Heat, Anoxia, Salt, Osmotic stress, High light, Oxidative stress	Nishizawa et al., 2006; Ogawa et al., 2007
	VviHsfA3a	VIT_08s0007g03900	2159	61.45	uniquely mapping	99.91	Drought, Salt, Heat, Cold, Oxidative stress, UV-B, Wounding: via DREB2A	Yoshida et al., 2008; Winter et al., 2007
	VviHsfA4a	VIT_10s0003g01770	2159	46.00	uniquely mapping	99.03	Heat, Salt, Osmotic stress, Cold, Biotic stress, Anoxia	Pérez-Salamó et al., 2014
	VviHsfA4b	VIT_12s0028g01410	2514	50.10	uniquely mapping	99.40	Cold stress	Tang et al., 2016
	VviHsfA5a	VIT_05s0029g00350	359	54.54	multiple hits	95.56	Represses HsfA4	Baniwal et al., 2007
	VviHsfA6a	VIT_00s0179g00150	368	40.91	uniquely mapping	100.00	Drought, Salt stress, Heat	Hwang et al., 2014
	VviHsfA6b	VIT_05s0020g04090	-	40.07	-	-	Salt stress, Cold, Osmotic stress	Von Koskul-Döring et al., 2007; Hwang et al., 2014
	VviHsfA8a	VIT_01s0011g05970	2198	48.06	uniquely mapping	99.71	Oxidative stress	Taki et al., 2005
	VviHsfA9a	VIT_11s0016g02010	1583	45.06	putative chimeras	99.72	Not induced by stress	Kotak et al., 2007
B	*VviHsfB1a*	VIT_07s0031g00670	2057	31.79	uniquely mapping	100.00	Oxidative stress, Biotic stress, Heat	Ikeda et al., 2011; Kumar et al., 2009; Taki et al., 2005
	VviHsfB2a	VIT_16s0100g00720	1521	29.26	uniquely mapping	99.46	Heat, High light	Wunderlich et al., 2014; Nishizawa-Yokoi et al., 2011; Charng et al., 2007; Kumar et al., 2009
	VviHsfB2b	VIT_02s0025g04170	3321	33.87	uniquely mapping	98.80	Heat, Biotic stress	Charng et al., 2007; Kumar et al., 2009
	VviHsfB3a	VIT_08s0007g08750	1942	27.78	putative chimeras	96.62	Oxidative stress, Water deficit	Taki et al., 2005; Soares-Cavalcanti et al., 2012
	VviHsfB4a	VIT_06s0009g02730	1332	33.07	uniquely mapping	99.67	Heat (decreases expression)	Qiao et al., 2015; Giorno et al., 2012
	VviHsfB4b	VIT_18s0001g10380	-	40.18	-	-	Heat (decreases expression)	Giorno et al., 2012
	VviHsfB5a	VIT_10s0597g00050	909	23.00	uniquely mapping	99.25	Ethylene, Methyljasmonate treatment	Hu et al., 2015
C	*VviHsfC1a*	VIT_11s0016g03940	1288	37.32	putative chimeras	99.87	Heat, Oxidative stress, Cold, Salt, Osmotic stress	Qiao et al., 2015; Taki et al., 2005; Miller and Mitler, 2006; Swindell et al., 2007; Zhang et al., 2015

5.3.2 The effect of elevated light exposure on the expression of *VviHsfs* genes in developing grape berries

5.3.2.1 Differential expression analysis of *VviHsf* genes in response to development and/or elevated light

To explore the specific expression patterns of the 19 *VviHsfs* in response to elevated light, a hierarchical clustering analysis was performed. In Figure 5.1, we report on six of these expression profile clusters. Broadly summarized, the expression of these 19 genes could be divided into two main clusters of which the first (A) represented genes that were expressed at low levels during early berry development until véraison, followed by higher levels of expression in ripening berries. Conversely, the second main cluster (B) represents genes that were expressed at lower levels after véraison compared to the expression of these genes during early berry development (EL31 and EL33).

A closer inspection of each of these broad clusters revealed several sub-clusters that further represented genes that were similarly affected by elevated light. An example of one of these sub-clusters whose members remained mostly unaffected by light exposure was cluster A3 in which these genes were expressed at very low levels during the green developmental stages, followed by higher expression levels from véraison onwards in control and exposed grapes. Contrastingly, sub-cluster B2 represented genes that were expressed at relatively high levels during the first two developmental stages compared to their expression after véraison in control grape samples, however, these genes were consistently expressed at higher levels in exposed compared to control grapes. These genes (*VviHsfA2a, VviHsfA6a* and *VviHsfB2a*) were therefore more strongly affected by the elevated light treatment, irrespective of the developmental pattern.

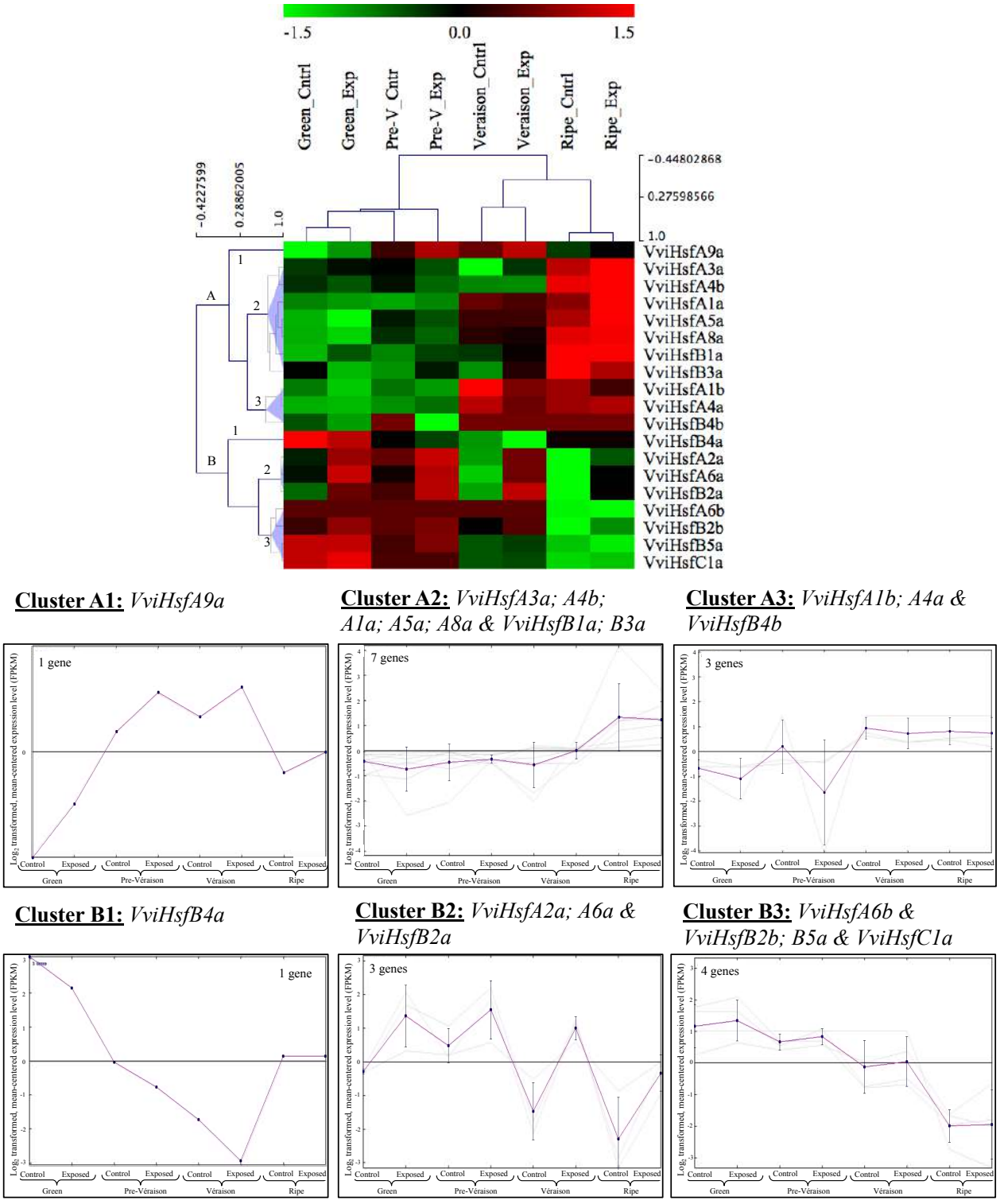


Figure 5.1. Hierarchical clustering analysis results representing the $\log_2$, mean-centered expression patterns of the nineteen *VviHsf* genes in grape berries at the green (EL31), Pre-Véraison (Pre-V; EL33), Véraison (EL35) and Ripe (EL38) developmental stages under control and exposed conditions.

To confirm the statistical significance of the differential expression induced by light exposure, the expression of the *VviHsfs* was compared at each phenological stage. The $\log_2$ fold changes between exposed and control samples are indicated in Table 5.2, alongside the level of significance for each of the statistically

differentially expressed genes ($q \leq 0.05$). These data provided an overview of which *Hsfs* responded most significantly to elevated light exposure and revealed that ten of the characterized *VviHsfs* were not significantly affected by elevated light at any of the developmental stages. Among the nine genes that were significantly upregulated during at least one of the developmental stages, *VviHsfA2a*, *VviHsfA6a* and *VviHsfB2a* (cluster B2 in Figure 5.1). were highly significantly upregulated throughout the entire berry development in response to elevated light.

Table 5.2. Table representing the nine *VviHsf* genes that show differential expression during at least one developmental stage when comparing control grapes to those that were exposed to elevated light at four developmental stages. Log_2FC represents the fold change (log_2) when comparing the FPKM expression values of each of the genes when comparing exposed to control samples. The level of significance of the differential expression is indicated by a *q*-value: * $q<0.05$; ** $q<0.01$; *** $q<0.001$

		Green		Pre-Véraison		Véraison		Ripe	
Gene name	Gene ID	Log_2FC	*q*-value	Log_2FC	*q*-value	Log_2FC	*q*-value	Log_2FC	*q*-value
VviHsfA1b	VIT_16s0039g01840	-0.24		-0.12		-0.38	**	-0.28	
VviHsfA2a	VIT_04s0008g01110	2.04	***	1.13	***	3.02	***	2.21	***
VviHsfA3a	VIT_08s0007g03900	0.28		-0.47		1.34	**	0.62	*
VviHsfA6a	VIT_00s0179g00150	2.26	***	1.71	***	3.27	***	2.81	***
VviHsfB1a	VIT_07s0031g00670	0.51	**	0.36	*	0.33	*	-0.07	
VviHsfB2a	VIT_16s0100g00720	0.65	***	0.35	*	1.11	***	0.88	***
VviHsfB2b	VIT_02s0025g04170	0.38	*	0.14		0.35	*	0.96	***
VviHsfB3a	VIT_08s0007g08750	-2.50		1.67		2.51		-1.78	**
VviHsfB4a	VIT_06s0009g02730	-0.92	**	-0.72		-1.21		0.00	

The expression of *VviHsfA1b* was only significantly affected at véraison, whereas the other *HsfA1* gene, *VviHsfA1a* (Cluster A2), remained unaffected by the treatment. Two of the other members of Cluster A2, *VviHsfB3a* and *VviHsfB4a,* as well as *VviHsfA3a* (Cluster B1) showed significantly altered expression when comparing exposed to control grapes at various developmental stages, however, these genes show negligibly low expression levels in developing grape berries (FPKM<1.0) and differential expression may appear statistically significant although the biological differences are subtle and possibly insignificant.

5.3.2.2 Identification of genes co-expressed with *VviHsf* genes in response to elevated light exposure

By identifying genes with known functions that are co-expressed with specific *VviHsfs*, general inferences could be made regarding the possible functions that the expression of these *Hsfs* may fulfill during berry acclimation to elevated light stress. The genes contained within cluster B2 (Figure 5.1) were therefore chosen for co-expression analysis because of their consistent upregulation in response to elevated light throughout berry development. Subsequently, *VviHsfA2a, VviHsfA6a* and *VviHsfB2a* were individually targeted for the identification of other genes that respond similarly to elevated light. These analyses yielded 181, 146 and 38 genes respectively co-expressed within the 90th percentile to *VviHsfA2a, VviHsfA6a* and

VviHsfB2a. In order to further determine whether these three *Hsfs* potentially share regulatory roles, the genes putatively co-expressed with each of these *Hsfs* were compared in a Venn diagram (Figure 5.2). Interestingly, despite showing similar expression patterns, *VviHsfB2a* only shared three co-expressed genes with both *VviHsfA2a* and *VviHsfA6a* with a further 12 co-expressed genes shared between *VviHsfA2a* and *VviHsfB2a*. Among these 12 shared genes were several Hsp genes that included a *Hsp70* gene (VIT_06s0004g04470). Unexpectedly, the three genes co-expressed with all three *VviHsfs* investigated did not code for heat shock proteins but rather encoded a thylakoid luminal protein (VIT_02s0154g000400), an unspecified short activator peptide motif (Aha1-domain) containing protein (VIT_08s0007g06710) and a CCR4-NOT transcription complex protein (VIT_09s0070g00060). GO enrichment analysis of all the genes putatively co-expressed with *VviHsfB2a* revealed that these genes are involved in several mechanisms of response to high light intensity, heat and the presence of reactive oxygen species (Figure 5.2). The genes represented in the Venn diagram are listed in Table C5.2; Addendum C of Chapter 5.

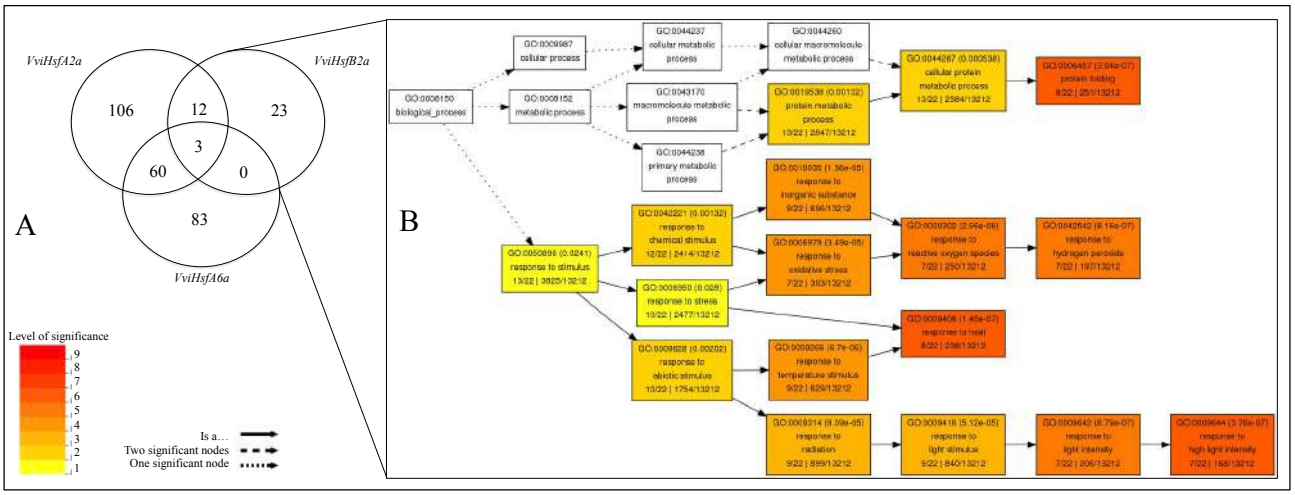


Figure 5.2. Co-expression analysis of the three Hsf genes (*VviHsfA2a, VviHsfA6a* and *VviHsfB2a*) that show consistent significant upregulation (q<0.05) in response to elevated light throughout berry development. A: Venn diagram depicting the number of shared genes that are co-expression with *VviHsfA2a*, *VviHsfA6a* and *VviHsfB2a*. B: GO Enrichment analysis of the 38 genes that are putatively co-expressed with *VviHsfB2a* under control and elevated light conditions throughout berry development.

Despite showing consistent upregulation in green grapes, neither *VviHsfA2a* nor *VviHsfA6a* were identified among the genes co-expressed with *VviHsfB2a*, whereas *VviHsfA2* was co-expressed with *VviHsfA6a* and vice versa. Furthermore, *VviHsfA2* and *VviHsfA6a* shared a much larger number (63) of putatively co-expressed genes that warranted further investigation. All the genes putatively co-expressed with both *VviHsfA2a* and *VviHsfA6a* were therefore compared in a separate Venn diagram (Figure 5.3). Genes that were simultaneously co-expressed with both *VviHsfA2a* and *VviHsfA6a* will further be referred to as "shared genes" for simplification purposes.

GO annotations revealed that a substantial percentage of each of these co-expressed gene groups was represented by *Hsps* and other molecular chaperones with the highest percentage (32%) within the shared gene group and the genes co-expressed with *VviHsfA2* (13%) (Figure 5.3A). These high percentages of Hsps and chaperones in each of these categories were dominated by low molecular weight *Hsps* (sHsps) and several *Hsp81* genes were also co-expressed either uniquely or simultaneously with *VviHsfA2a* and *VviHsfA6a*. Furthermore, among the *VviHsfA2* co-expressed genes and the shared genes were several encoding *Hsp101*, *Hsp70* and a large number of chaperonins. It is further important to note that the only Hsf gene representing the 6% of Hsfs uniquely co-expressed with *VviHsfA2a* was in fact *VviHsfA6a* and vice versa for *VviHsfA6a* and *VviHsfA2a*, as previously mentioned.

In order to create a holistic view of the functional association of the genes either uniquely or simultaneously co-expressed with *VviHsfA2a* and *VviHsfA6a*, GO enrichment analysis was performed, followed by the summarizing of the resulting enrichment data using ReviGO. The ReviGO summary graphs representing each of the co-expressed gene groups are represented in Figure 5.3B. Although several GO terms were similarly enriched for each of these gene groups as could be expected, there were uniquely enriched GO terms that were more specific and revealing with regards to the possible functional roles of these Hsfs in grape berries.

The shared group of genes was associated with general responses associated with plant abiotic stress response such as simultaneous responses to oxidative stress, high light intensity, heat, endoplasmatic reticulum stress, and the presence of hydrogen peroxide, while secondary metabolism and protein folding mechanisms were similarly upregulated. In contrast, the genes putatively co-expressed with either *VviHsfA2a* or *VviHsfA6a* responded more specifically to elevated exposure to light. The enriched GO terms associated with these two distinct gene sets distinguished clearly between the perception of specific abiotic stresses as indicated by the fact that both of these gene groups were associated with the berries' response to light intensity and not temperature. Both of these sets of uniquely putatively co-expressed genes were functionally associated with the light reaction of photosynthesis, protein folding and response to reactive oxygen species, but the genes co-expressed with *VviHsfA6a* show further distinct characteristics as well. The 83 genes putatively co-expressed with this *Hsf* were also functionally enriched for GO terms associated with pigment metabolism, isoprenoid metabolism, thylakoid membrane organization and plastid organization. Some of the more interesting genes uniquely putatively co-expressed with *VviHsfA6a* included genes encoding enzymes associated with plant stress attenuation like a Bax inhibitor protein encoding gene (VIT_07s0151g00280), two glutathione-S-transferases (VIT_05s0049g01080, VIT_07s0005g00030) and a 2-oxo-glutarate Fe(II) oxygenase family gene (VIT_03s0063g01310). Furthermore, many genes involved in either photosynthesis or chloroplast biosynthesis were represented in this gene group and included a photosystem II stability/assembly factor (VIT_01s0011g02150), a PsbS encoding gene (VIT_18s0001g02740) and several chloroplast precursor protein encoding genes (VIT_18s0122g00960, VIT_12s0035g01080, VIT_18s0001g10460). The complete gene lists putatively co-expressing either

uniquely or simultaneously with *VviHsfA2a* and *VviHsfA6a* are available in Table C5.2; Addendum C of Chapter 5.

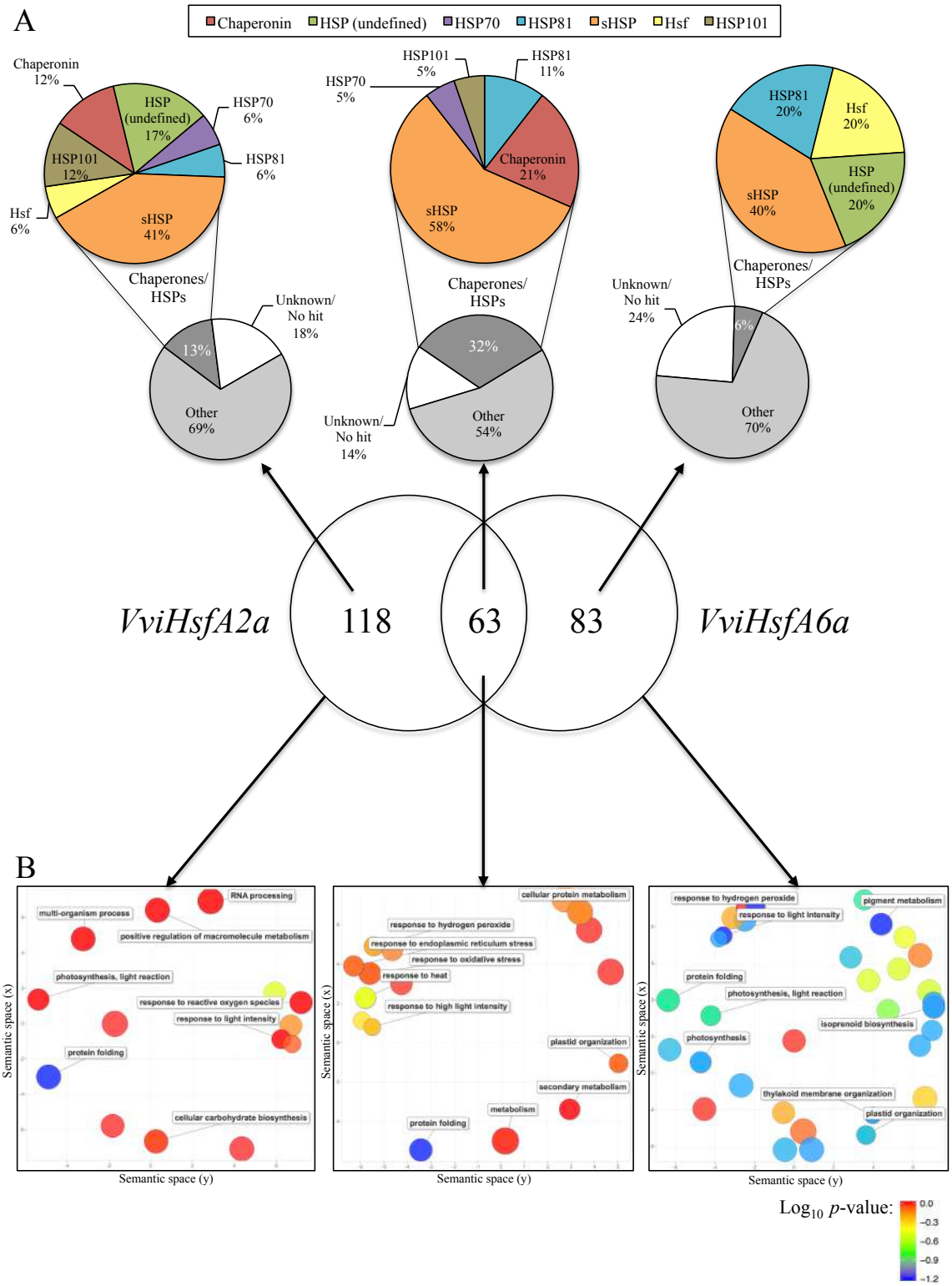


Figure 5.3. Venn diagram representing the number of genes either uniquely or simultaneously co-expressed with *VviHsfA2a* and *VviHsfA6a*. A: Pie charts depicting the percentage (%) of specific Hsp encoding genes putatively co-expressed with either *VviHsfA2a*, *VviHsfA6a* or shared between both groups. B: GO enrichment analysis and subsequent summarization of significantly enriched GO terms (p<0.05) representing each group of co-expressed genes.

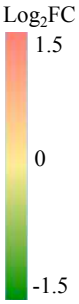
5.3.3 The expression of 'Module 17' genes in grapes responding to elevated light

Differential expression analysis provided results in order to determine which of the module 17 genes were significantly altered (q<0.05) by elevated light throughout grape berry development (Table 5.3). The results from this investigation revealed that every gene in module 17 was highly significantly upregulated from early berry development (EL31) until véraison (EL35), when comparing exposed to control grapes. When the berries achieved ripeness at EL38, only seven of these genes were no longer significantly upregulated by elevated light.

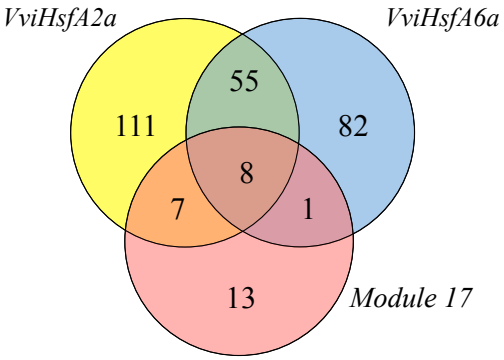
A Venn diagram, comparing the module 17 genes with the genes either uniquely or simultaneously co-expressed with *VviHsfA2a* and *VviHsfA6a* (Figure 5.4) showed that sixteen genes within module 17 were also putatively co-expressed either uniquely or simultaneously with *VviHsfA2a* and *VviHsfA6a* (Figure 5.4A). Eight of these genes were putatively co-expressed with both *Hsfs* investigated and include six *Hsps* well known for their involvement in plant stress response (Figure 4B; VIT_17s0000g07190, VIT_18s0041g01230, VIT_16s0050g01150, VIT_13s0047g00110, VIT_19s0085g01050, VIT_01s0010g02290). Another seven of the module 17 genes were uniquely co-expressed with *VviHsfA2a*, whereas only one other *Hsp17.1* encoding gene (VIT_13s0019g03160) was putatively expressed with *VviHsfA6a*. Interestingly, among the thirteen module 17 genes that were not putatively co-expressed with the two *VviHsfs* compared in the Venn diagram (Figure 5.4A), five genes were putatively co-expressed with other class b *Hsf* genes differentially expressed in response to elevated light (Figure 5.4B).

Table 5.3. The effect of elevated light exposure on the expression of the 29 genes identified in 'Module 17' (Liang et al., 2014) in Sauvignon Blanc grapes at four phenological stages. Differential expression is indicated as a $\log_2$ fold change value when comparing exposed to control grapes at each developmental stage. Non-significant differential expression (q>0.05) is indicated by a bold contoured frame.

		Log$_2$ Fold-Change (Exposed vs Control)			
Gene name	Gene ID	EL31	EL33	EL35	EL38
Armadillo/beta-catenin repeat	*VIT_08s0007g00740*	1.07339	1.11207	1.30564	0.321133
Galactinol synthase	*VIT_07s0005g01980*	5.08813	2.82536	4.0672	2.75795
Heat shock 22 kDa protein	*VIT_16s0022g00510*	1.58781	1.08104	2.60622	1.18491
Heat shock cognate 70 kDa protein 1	*VIT_06s0004g04470*	2.45128	1.4292	2.55339	0.827591
Heat shock cognate 70 kDa protein 1	*VIT_08s0007g00130*	0.750107	0.634158	1.28783	0.871692
Heat shock protein 101	*VIT_17s0000g07190*	2.09606	1.48337	2.97594	1.53876
Heat shock protein 17.4 kDa class I	*VIT_06s0004g05770*	3.43425	2.29061	2.89292	1.66319
Heat shock protein 18.1 kDa class I	*VIT_13s0019g03160*	4.26578	3.1326	4.07101	1.89582
Heat shock protein 26a, chloroplast	*VIT_01s0010g02290*	4.03879	2.84299	4.57729	2.09236
Heat shock protein 26a, chloroplast	*VIT_16s0098g01060*	1.51639	3.96766	3.74977	5.05174
DnaJ homolog, subfamily B, member 4	*VIT_10s0003g00260*	1.37122	0.972413	1.7724	0.796953
DnaJ homolog, subfamily B, member 9	*VIT_08s0217g00090*	1.57543	1.49294	1.61574	0.65532
Heat shock protein 70	*VIT_18s0041g01230*	1.48425	1.29615	1.82012	0.255823
Heat shock protein 70	*VIT_05s0020g03330*	2.09121	1.67934	2.19157	0.79702
Heat shock protein 81-1	*VIT_16s0050g01150*	3.93571	2.53792	5.15296	2.09651
Heat shock protein Cytosolic class II low	*VIT_04s0008g01590*	5.78991	3.42476	5.49496	2.90819
Heat shock protein Cytosolic class II low	*VIT_04s0008g01490*	3.25254	2.48149	3.45939	1.5886
Heat shock protein HSP17	*VIT_19s0085g01050*	5.34646	3.57898	5.98274	4.56156
Small heat-shock protein HSP17.5 Cytos	*VIT_13s0019g00860*	3.33741	2.38195	3.10281	1.40919
Heat shock protein HSP22.7	*VIT_12s0035g01910*	2.56943	1.85591	1.12899	1.40582
Heat shock protein MTSHP	*VIT_02s0154g00480*	3.33909	2.2463	4.28941	2.20284
Heat shock protein precursor 22.0 kDa cl	*VIT_18s0089g01270*	4.33008	1.9431	3.43595	2.49672
Multiprotein-bridging factor 1c MBF1C	*VIT_11s0016g04080*	2.11279	1.06054	2.31336	-0.0567945
Rab/Ypt GTPase Ara4-interacting protein	*VIT_02s0025g04060*	2.37147	1.98722	2.58605	1.19695
Ripening regulated protein DDTFR8	*VIT_13s0047g00110*	2.36303	1.85365	2.97649	0.234527
Transformer serine/arginine-rich ribonuc	*VIT_06s0004g06010*	0.905745	0.884985	1.16293	0.274662
Unknown protein	*VIT_07s0185g00040*	1.31774	1.23119	1.31523	0.413696
Unknown protein	*VIT_17s0000g00070*	1.20129	2.84987	2.52206	0.463622
Unknown protein	*VIT_11s0078g00260*	0.561118	0.52759	1.21582	0.688514



A



B

Functional annotation (Grimplet et al., 2012)	Gene ID	*VviHsf* co-expressed
Heat shock protein 101	*VIT_17s0000g07190*	*VviHsfA2a, VviHsfA6a*
heat shock protein 26a, chloroplast	*VIT_01s0010g02290*	*VviHsfA2a, VviHsfA6a*
Heat shock protein 70	*VIT_18s0041g01230*	*VviHsfA2a, VviHsfA6a*
heat shock protein 81-1	*VIT_16s0050g01150*	*VviHsfA2a, VviHsfA6a*
heat shock protein Cytosolic class II low molecular weight	*VIT_04s0008g01490*	*VviHsfA2a, VviHsfA6a*
heat shock protein HSP17	*VIT_19s0085g01050*	*VviHsfA2a, VviHsfA6a*
Multiprotein-bridging factor 1c MBF1C	*VIT_11s0016g04080*	*VviHsfA2a, VviHsfA6a*
Ripening regulated protein DDTFR8	*VIT_13s0047g00110*	*VviHsfA2a, VviHsfA6a*
armadillo/beta-catenin repeat	*VIT_08s0007g00740*	*HsfA2a*
galactinol synthase	*VIT_07s0005g01980*	*HsfA2a*
Heat shock 22 kDa protein	*VIT_16s0022g00510*	*HsfA2a*
heat shock cognate 70 kDa protein 1	*VIT_06s0004g04470*	*HsfA2a*
heat shock protein 17.4 kDa class I	*VIT_06s0004g05770*	*HsfA2a*
heat shock protein Cytosolic class II low molecular weight	*VIT_04s0008g01590*	*HsfA2a*
Rab/Ypt GTPase Ara4-interacting protein	*VIT_02s0025g04060*	*HsfA2a*
DnaJ homolog, subfamily B, member 4	*VIT_10s0003g00260*	*VviHsfB2a*
heat shock protein MTSHP	*VIT_02s0154g00480*	*VviHsfB2a*
Unknown protein	*VIT_07s0185g00040*	*VviHsfB2a*
Unknown protein	*VIT_11s0078g00260*	*VviHsfB2a*
heat shock cognate 70 kDa protein 1	*VIT_08s0007g00130*	*VviHsfB2b*
heat shock protein 26a, chloroplast	*VIT_16s0098g01060*	-
DnaJ homolog, subfamily B, member 9	*VIT_08s0217g00090*	-
Heat shock protein 70	*VIT_05s0020g03330*	-
small heat-shock protein HSP17.5 Cytosolic class I	*VIT_13s0019g00860*	-
heat shock protein HSP22.7	*VIT_12s0035g01910*	-
Heat shock protein precursor 22.0 kDa class IV	*VIT_18s0089g01270*	-
transformer serine/arginine-rich ribonucleoprotein	*VIT_06s0004g06010*	-
Unknown protein	*VIT_17s0000g00070*	-
heat shock protein 18.1 kDa class I	*VIT_13s0019g03160*	*HsfA6a*

Figure 5.4. *VviHsf* co-expression and 'Module 17' genes. A: Venn diagram comparing co-expressed genes with *VviHsfA2a* and *VviHsfA6a* and the 29 genes represented by 'Module 17'. B: Summary table of the 29 Module 17 genes, their functional annotations and the grapevine Hsfs they are co-expressed with throughout grape berry development.

5.3.4 Construction of a working model for the expression and putative regulation of *VviHsfs*

Based on the combined findings reported regarding *Hsf* regulation in *Arabidopsis* (recently reviewed in Yabuta, 2016), in conjunction with the findings reported in this study, we propose a working model of *Hsf* gene expression and putative regulation in grape berries in response to elevated light (and during acclimation) (Figure 5.5).

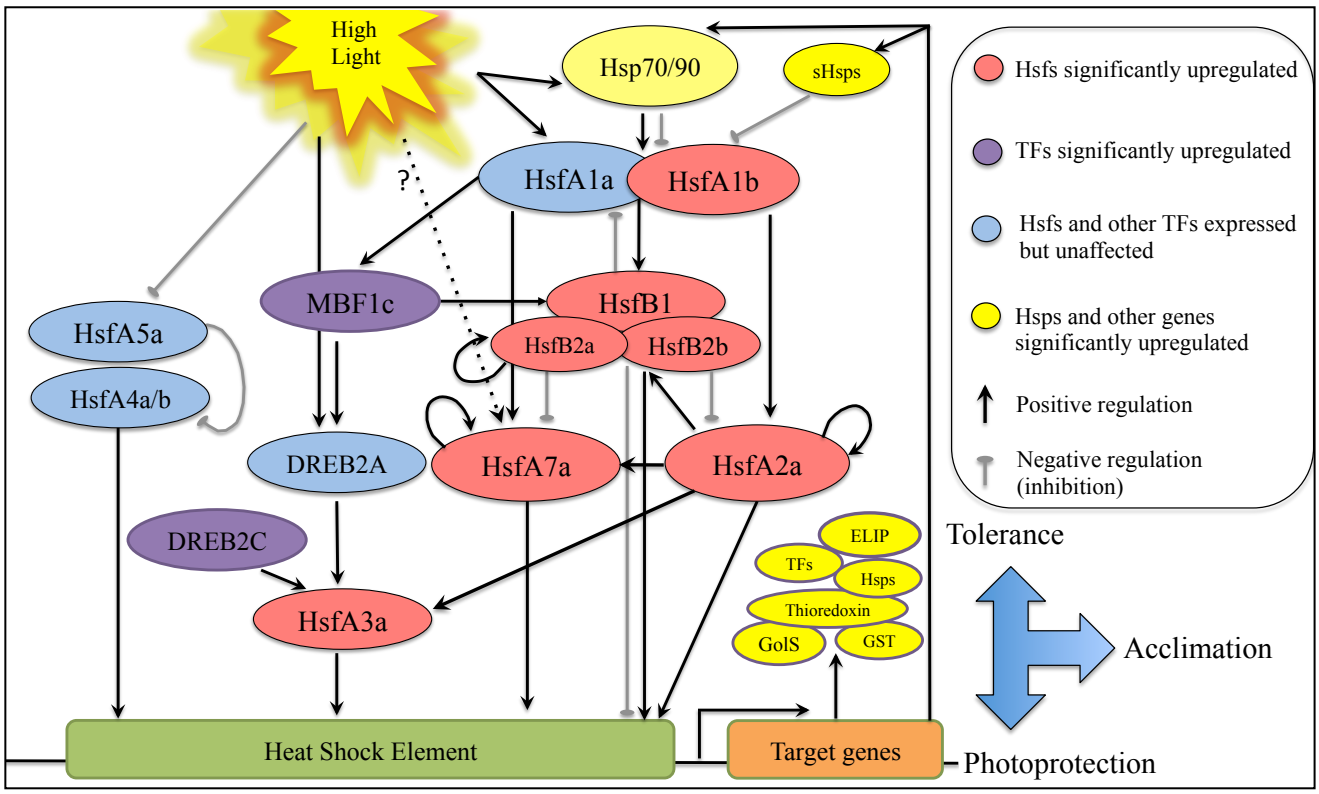


Figure 5.5. A simplified schematic representation of the impact of elevated light exposure on the many potential interactions that form part of the Hsf regulatory network in Sauvignon Blanc grape berries. Genes that are shown to overlap in the diagram are considered to be co-regulated and further co-regulates down-stream activities. DREB2, Dehydration Responsive Element Binding Protein 2; TFs, Transcription factors; GolS, Galatinol synthase; GST, Glutathione-S-transferase; Hsps, Heat shock proteins; Hsfs, Heat shock factors; sHsp, Small heat shock proteins; ELIP, Early light-inducible protein.

5.4 Discussion

The role of plant Hsfs are currently under investigation due to their involvement in plant responses to abiotic stress conditions, through their regulatory role in the expression of *Hsps* and the activation of several stress mitigation mechanisms. Recently, the genome-wide identification and characterization of these ubiquitous Hsf encoding genes have been reported in several plant species (Wei et al., 2016; Tang et al., 2016; Dossa et al., 2016; Hu et al., 2015; Liu et al., 2016; Song et al., 2014; Hu et al., 2016).

Interestingly, although grapevine (*V. vinifera)* is one of the most widely cultivated fruit crops worldwide, the expression patterns and the associated molecular mechanisms of grapevine *Hsf* genes have not been fully elucidated to our knowledge. In this report, we explored the expression patterns of the grapevine *Hsf* genes identified by Hu et al., (2016) to ultimately determine the effect that elevated light exposure has on the expression of these genes in developing Sauvignon Blanc grape berries.

5.4.1 The functional associations of *Hsfs*

The mechanism of *Hsf* activation has been extensively studied and recently reviewed (Ohama et al., 2017). Under non-stressed conditions, similar to the results presented here, *HsfA1* genes are frequently constitutively expressed while the proteins they encode remain in inactive forms, bound to Hsp90 and Hsp70 in the cytosol (Sugio et al., 2009; Hahn et al., 2011). Under these normal conditions, damaged and misfolded proteins are polyubiquitinated and subsequently degraded. However, when ROS accumulates in response to adverse growing conditions, for example light stress, the accumulation of degraded proteins accelerates. This accumulation is the result of the simultaneous acceleration of protein damage and the inhibition of the 26S proteasome responsible for the degradation of damaged proteins induced by stress. The chaperone activity of Hsps are subsequently required to facilitate the turnover of damaged proteins and hereby, Hsp90 and Hsp70 are sequestered from their inactive complex with HsfA1. As a result, HsfA1 is released and is therefore free to activate a wide range of target genes that include several other *Hsfs*, including *HsfA2*, *Hsps* and other genes involved in plant stress mitigation.

HsfA1s are frequently referred to as the ‘master regulators’ of plant abiotic stress response. While several HsfA1s have been extensively characterized in the context of abiotic stress response, the expression of *HsfB1* has been implicated in the suppression of the genes involved in heat stress response under normal conditions in *Arabidopsis* (Ikeda et al., 2011) and is known to repress the expression of other *Hsfs* such as *HsfA1s* and *HsfA2* through feedback inhibition (Ikeda et al., 2011). Additionally, the activity of *HsfB1s* create a primed “standby” state under non-stressed conditions, more adequately preparing the plant for abiotic stresses that may arise (Pick et al., 2012). The simultaneous high levels of expression of the *VviHsfA1s* and *VviHsfB1* therefore potentially point towards a transcriptional homeostasis between the activation and suppression of the expression of abiotic stress related genes while normal growth and development is maintained (from the expression patterns found in the control samples). These findings were further supported when the expression of these genes were also evaluated in other grapevine tissues/organs as well as in a panel of different cultivars by mining published datasets (Refer to section B5.2 and Figures B5.3 & B5.4; Addendum B of Chapter 5).

Although not as frequently reported, *HsfA2* is also known to be involved in cell differentiation and proliferation associated with growth and development under non-stress conditions as reported in this investigation (Figure 5.1). For example, under normal conditions, the expression *HsfA2* is highly induced in

the development of tomato anthers (Giorno et al., 2010) and the formation of *Arabidopsis* callus and shoot development in tissue culture explants (Che, 2002). Our analysis of selected available datasets (Fasoli et al., 2012; Massonnet et al., 2017) further revealed tissue specific expression of *VviHsfA2a* limited to mostly vegetative, photosynthesizing tissues, with very low or undetectable expression levels in mature, woody or senescing tissues (Figure B5.1; Addendum B of Chapter 5). These tissues included senescing leaves, roots or any berry tissues (including seeds) after véraison, when the growth of the grapes begins to cease, hereby further contributing to our knowledge regarding the role of *HsfA2* in cell differentiation and growth of plant tissues under normal growing conditions. Interestingly, besides *VviHsfA2a*, only three other class b *VviHsfs* (*VviHsfB4a, VviHsfB4b* and *VviHsfB5a*) displayed similarly low expression levels in mature woody stems and dormant winter buds. All other *VviHsfs* were therefore expressed at various levels in these tissues, hereby underpinning the critical roles that the expression of these genes plays in the protection of metabolically stagnant tissues under normal conditions.

HsfA2 is the most characterized Hsfs in plants and has been reported to accumulate rapidly in response to heat shock, high light stress, salinity and other oxidative stresses (Charng et al., 2007; Miller & Mittler, 2006; Nishizawa et al., 2006; Ogawa et al., 2007; Schramm et al., 2006) and has been established as a signal enhancer for the activity of HsfA1 (Scharf et al., 1998; Chan-Schaminet et al., 2009). HsfA2 is further known to activate the expression of not only itself (Liu et al., 2013) but also other *Hsfs* termed 'transcriptional relay' Hsfs by Jacob et al., (2017). These Hsfs include HsfA3 and HsfA7a that are considered to be the dominant activators of the expression of *Hsps* during plant stress recovery and have been established as the Hsfs responsible for the so-called 'heat-acclimation phenotype' (Charng et al., 2007; Nishizawa et al., 2006; Schramm et al., 2006). Furthermore, because class a and class b Hsf compete for the same or similar promoter binding sights, class b Hsfs, such as HsfB2a, have been proposed to play a role in attenuation of strongly induced expression of *HsfA2* and *HsfA7a* during abiotic stress. This tight-knit homeostasis between the expression of class a and class b Hsfs have been well established under abiotic stress conditions and the plant's ability to acclimate to these stresses.

What our comparative investigation of the grapevine Hsf-encoding genes further established, is the remarkably conserved nature of the expression of these genes when comparing other *V. vinifera* genotypes, regardless of the possible impacts of variable experimental systems and their potential effects on the transcriptional results. In this investigation, the expression of the nineteen *VviHsfs* were compared from two separate Sauvignon Blanc RNASeq datasets, a large-scale Nimblegen microarray analysis performed on the red cultivar, Corvina (Fasoli et al., 2012) and RNASeq data generated from five white and five red cultivars harvested from various regions throughout Italy (Massonnet et al., 2017) that were inevitably grown under highly variable conditions. Subtle expression differences between Sauvignon Blanc and the other cultivars could be identified for *VviHsfA4a* and *VviHsfA8a* (Figure B5.2; Addendum B of Chapter 5) that could point towards a difference in abiotic stress responses between these cultivars, since these Hsfs have been implicated in reactive oxygen sensing in *Arabidopsis* (Davletova, 2005).

5.4.2 *VviHsfA6a* may be misannotated as its syntenic gene, *HsfA7a.*

The expression and activity of *HsfA6a* has only been extensively characterized in *Arabidopsis* by Hwang et al., (2014). The authors reported that the expression of *HsfA6a* is induced by abscisic acid (ABA), salinity and other dehydration stresses and that high temperatures did not induce its expression. Aside from one earlier study that also reported the expression of *HsfA6a* in response to cold stress in *Arabidopsis* (Koskull-Döring et al., 2007), subsequent studies mostly supported the fact that *HsfA6a* expression is induced predominantly by ABA, salt, drought and osmotic stresses (Huang, et al., 2014, 2016; Hwang et al., 2014). Hwang et al., (2014) further reported that the *HsfA6a* promoter contains ABA-responsive elements that include AREB1 and AREB3, as well as ABF3 and that overexpression of this *Hsf* resulted in *Arabidopsis* mutants highly sensitive to ABA with elevated resistance to salt and dehydration stress. In order to confirm the involvement of *VviHsfA6a* in response to the elevated light treatment implemented in this investigation, these *AREB* and *ABF* encoding genes were targeted in the RNASeq data generated in this study. This investigation surprisingly revealed that these ABA-responsive genes were not upregulated in response to the treatment (Table C5.1; Addendum C of Chapter 5). Although ABA concentrations of the berry samples were not measured, it was established that the levels of the xanthophyll precursor for ABA synthesis, neoxanthin, was not higher in exposed grapes, nor were the genes involved in ABA synthesis significantly altered (Young et al., 2016). Although the possibility exists that ABA may have been transported from other grapevine source organs that may have stimulated the upregulation of *VviHsfA6a*, the expression pattern of this gene does not support this idea (Figure 5.1). In developing grapes, ABA levels are known to be low until véraison, after which it increases to participate in the modulation of ripening processes (reviewed in Serrano et al., 2017). *VviHsfA6a* expression, however, increased before véraison, after which it decreased dramatically until the grapes were ripe under normal (control) conditions (Figure 5.1). Sufficient levels of ABA required for the activation of *VviHsfA6a* would therefore be highly unlikely in order to induce the expression pattern reported for this gene under elevated light conditions (Cluster B2; Figure 5.1). The lack of any other publications reporting *HsfA6a* upregulation in response to light further supports this notion.

Furthermore, in grapevine leaves, a study focused towards genes highly sensitive to high light exposure identified and named a gene *HsfA7a* in the grapevine genome that was among the top 10 most significantly upregulated grapevine genes in response to high light (Carvalho et al., 2011). The accession provided for this *HsfA7a* (Carvalho et al., 2011) revealed that this *HsfA7a* and the *VviHsfA6a* included in this study (based on Hu et al., 2016) was the same in the NCBI database.

Although the authors responsible for the characterization of the grapevine Hsf genes acknowledged that *VviHsfA6a* and *HsfA7a* from *Arabidopsis* were syntenic genes, they did not report the presence of a *VviHsfA7a* orthologue in the grapevine genome. Our analysis suggests that the *VviHsfA6a* reported by Hu et al., (2016) was in fact *VviHsfA7a*. This is supported by the fact that *HsfA6a* is known to activate the expression of *HsfA6b*, which was not the case in our investigation. Furthermore, the simultaneous

upregulation of *HsfA2, HsfA7a* and *HsfB2a* have been extensively reported in other plant systems under various environmental stress conditions (Busch et al., 2005; Ma et al., 2015; Nishizawa-Yokoi et al., 2011; Charng et al., 2007; Kissen et al., 2016; Koskull-Döring et al., 2007; Larkindale et al., 2008; Sugio et al., 2009; Ikeda et al., 2011; Nishizawa et al., 2006; Liu et al., 2011; Aparicio et al., 2009; Wu et al., 2015), similar to the results presented in this study. In this report, we will further refer to this highly upregulated Hsf as *VviHsfA7a*.

5.4.3 Three *VviHsfs* interact to activate and maintain abiotic stress response mechanisms for effective berry acclimation to elevated light exposure

Differential expression analysis revealed three *VviHsfs* that were significantly upregulated by elevated light throughout the entire developmental progression from the green stages until the berries were ripe. These *VviHsfs* (*VviHsfA2a, VviHsfA7a* and *VviHsfB2a*) were further explored in order to contextualize their involvement in berry acclimation to elevated light. Although the simultaneous expression of *HsfA2, HsfA7a* and *HsfB2a* has been reported in several other investigations focused towards abiotic stress responses in plants (Busch et al., 2005; Ma et al., 2015; Nishizawa-Yokoi et al., 2011; Charng et al., 2007; Kissen et al., 2016; Koskull-Döring et al., 2007; Larkindale and Vierling, 2008; Sugio et al., 2009; Ikeda et al., 2011; Nishizawa et al., 2006; Liu et al., 2011; Aparicio et al., 2009; Wu et al., 2015), to our knowledge, the expression of these genes have not been explored in grapevine. In tomato, it was shown that a physical interaction exists between constitutively expressed *HsfA1* and abiotic stress-induced *HsfA2* (Chan-Schaminet et al., 2009). These Hsfs together form a super activation complex that induces the expression of *Hsps* and other stress-response genes at levels up to five fold higher than when active separately. However, in *Arabidopsis*, transient reporter assays revealed that the expression of the HsfA1 isomers, *HsfA1d* and *HsfA1e*, directly activate the expression of *HsfA2* (Nishizawa-Yokoi et al., 2011). Furthermore, the knockout mutants of HsfA1d/A1e not only suppressed the expression of *HsfA2* but other Hsfs, including *HsfA7a* and *HsfB2a*, as well. These knock-out mutants further showed decreased activity of photosystem II under high light stress conditions, hereby indicating that *HsfA1d* and *HsfA1e* are the key regulators responsible for the Hsf signaling network during adverse environmental conditions.

Regardless of the fact that the presence of *HsfA1d* and *HsfA1e* were not detected in the grapevine genome (Hu et al., 2016), *VviHsfA2* was most significantly upregulated by elevated light throughout the entire development. Although Nishizawa-Yokoi et al., (2011) attributed the regulation of *HsfA2* to *HsfA1d/A1e* and not *HsfA1a/A1b*, double knockout mutants of *HsfA1d/A1e* were slightly sensitive to elevated light stress and *HsfA2* was not completely suppressed. Furthermore, double knockout mutants of *HsfA1a/A1b* were impaired in the expression of *HsfA7a* and *HsfB2a* during heat stress (Lohmann et al., 2004; Busch et al., 2005) and overexpression of *HsfA2* resulted in upregulation of *HsfB2a* expression (Ogawa et al., 2007). Furthermore, Liu et al., (2013) identified an autoregulatory feedback loop that allows some heat–shock induced Hsf splice-variants to induce expression of the same *Hsf* gene. Among the Hsf genes that were identified to be

self-inducible were *HsfA2, HsfA7a* and *HsfB2a* (Liu et al., 2013). Taken together these findings therefore revealed that other Hsfs might compensate for the role of *HsfA1d/A1e* that may explain the dramatic upregulation of *VviHsfA2a*, *VviHsfA7a* and *VviHsfB2a* in the absence of *VviHsfA1d/A1e* in grapevine.

Regardless of the wealth of information generated for the co-expression of *HsfA2, HsfA7a* and *HsfB2a*, the exact gene targets of *HsfA7a* remains to be established. The important role of *HsfA7a* in plant stress response is however irrefutable. For example, *Arabidopsis* knockout mutants of *HsfA7a* showed decreased thermotolerance (Larkindale and Vierling, 2008). It is widely assumed that *HsfA2a* and *HsfA7a* are possibly co-regulated by *HsfA1*, and that hereby these Hsfs share common functional properties. Although the data generated in our investigation supports this co-regulation hypothesis of *HsfA2* and *HsfA7a*, co-expression analysis results also revealed that *HsfA7a* might play a distinct role in photoprotection, independent of *HsfA2* and the expression of general abiotic stress-related *Hsp* expression. Not only were the genes uniquely co-expressed with *VviHsfA7a* under elevated light conditions significantly enriched in the functional categories associated with the biosynthesis of isoprenoids, pigments and the photosynthetic machinery, only one of these co-expressed genes were also present in Module 17 (Liang et al, 2014). Because the genes within Module 17 are associated with the general and consistent abiotic stress responses reported in numerous grapevine studies, this limited overlap of genes co-expressed with *VviHsfA7a* might indicate a more light-specific stress response in grape berries, facilitated by the upregulation of *VviHsfA7a*.

To further support this idea, only three genes were simultaneously co-expressed with *VviHsfA7a*, *VviHsfA2a* and *VviHsfB2a*, and none were shared between *VviHsfA7a* and *VviHsfB2a* regardless of the fact that these genes are supposedly co-regulated by *HsfA1a/A1b* and putatively compete for the same promotor gene targets in *Arabidopsis* (Nishizawa-Yokoi et al., 2011). This unique regulatory role of *VviHsfA7a* remains to be explored on the protein level and the identification of the direct gene targets of this Hsf will significantly enrich our current understanding of how plants acclimate to increased exposure.

It is however imperative to remain aware of the fact that co-expression analysis implemented in this study explores the correlation between certain genes and not causality. The fact that genes are co-expressed therefore does not imply that their expression has any specific influence on each other but simply that these genes are similarly regulated by the specific treatment at that developmental stage. These analyses however provided information to develop a working model for future studies focused towards targeting the causality of the expression of these *VviHsfs* under various abiotic stresses (Figure 5.5).

According to this model, HsfA1/A1b act as the master regulators involved in grape light stress response by activating the expression of various other transcription factors (TFs) that include *MBF1c* and the *Hsf* triad between *HsfA2a*, *HsfA7a* and the *HsfBs* that activates and attenuates the stress responses through several activation and suppression feedback loops. This *Hsf* triad was further recently shown to be self-inducing and

the data presented here further supports this discovery. Additionally, MBF1c further activates *HsfA3* via the expression of both *DREB2A* and *DREB2C*.

Among the many other targets of HsfA2a are *HsfA3* as well as a wide range of stress-related gene targets that contain the heat shock element (HSE) domain. Some of these target genes include other transcription factors that lead to the upregulation of various stress markers such as *thioredoxins*, *glutathione-S-transferases*, *GolS1*, *ELIP1* and a range of *Hsps* that collectively contribute to the tolerance, protection and acclimation strategies implemented by grapes to mitigate the potentially harmful effects of elevated light.

Additionally, we have uncovered a potentially novel role for *VviHsfA7a* in response to increasing light exposure in particular. This *Hsf* was putatively co-expressed with various genes associated with both antioxidant and sunscreening activities, independent of the upregulation of its general abiotic stress-related co-activator, *HsfA2a*. These findings may point towards a link between the upregulation of *VviHsfA7a* and the implementation of several photoprotective mechanisms in these developing grapes as previously reported (Chapter 3). Among these photoprotective mechanisms was the rapid turnover of the photosynthetic machinery proteins of the thylakoid membrane, as well as the accumulation of elevated levels of photoprotective carotenoids and flavonoid compounds with both antioxidant and sunscreening abilities (Young et al., 2016; Du Plessis et al., 2017). The identification of the direct gene targets of *VviHsfA7a* will shed light on its role in grape berry photoprotection. Furthermore, whether this induction of *HsfA7a* expression is directly a result of light exposure or indirectly via the activation by *HsfA1a/A1b* remains to be determined.

5.4.4 Conclusions and future prospects

In this study, the expression of *Hsfs* in *V. vinifera* was explored for the first time. This investigation identified three genes that were significantly upregulated by elevated light treatment throughout grape berry development and that one of these genes, named *VviHsfA6a* (Hu et al., 2016) in fact behaved as *HsfA7a* based on its sequence and putative functional associations as well. In support of what has been established regarding the regulation of Hsf expression in other plant systems, grapevine *VviHsfA2a* and *VviHsfA7a* appear to be regulated by either the constitutive expression of *VviHsfA1* at low levels or self-induced upon elevated exposure to light that has not been reported in previous investigations. Furthermore, a unique putative role for the expression of *VviHsfA7a* involved in photoprotection under elevated light conditions was identified, independent of *VviHsfA2* expression that warrants further investigation to ultimately contribute to our understanding of how grape berries acclimate to elevated light exposure. In this study, we proposed a putative working model representing the possible regulatory interactions between the various *Hsf* genes in response to elevated light in grape berries.

5.5 References

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Addendum A to Chapter 5

This Addendum contains relevant and additional data not shown in Chapter 5.

A5.1 Materials and Methods

A5.1.1 The *de novo* assembly of the Sauvignon blanc transcriptome.

A detailed description of the method pipeline including the data used, the digital normalization, the *de novo* assembly, clustering, redundancy removal and selection of the final transcripts are summarized below.

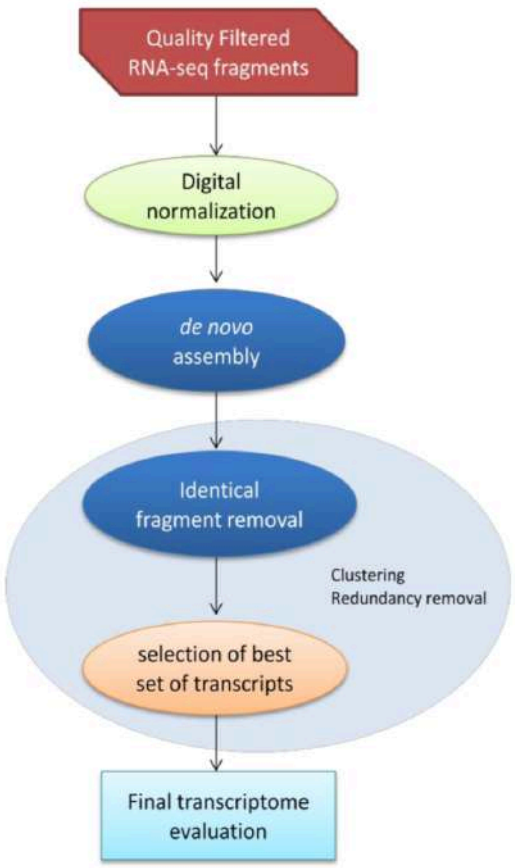

Figure A5.1. The *de novo* transcriptome assembly pipeline.

Table A5.4. Sequencing, filtering and starting data selection statistics for *de novo* assembly.

Sample_ID	Tissue	Stage	Treatment	Avg_Library_size (bases)	# Sequencing Fragments (100X2)	# Filtered fragments (100X2)	# Fragments selected for de novo assembly
153	berry	1	Exposed	407	37,270.320	31.819.740	10.000.000
162	berry	1	Exposed	365	29.691.861	25.849.345	10.000.000
164	berry	1	Exposed	409	61.412.802	43.641.861	10.000.000
253	berry	2	Exposed	396	45.555.091	37.889.256	10.000.000
262	berry	2	Exposed	471	29.595.812	25.907.066	10.000.000
264	berry	2	Exposed	354	43.603.478	37.284.005	10.000.000
353	berry	3	Exposed	452	28.664.640	23.729.795	10.000.000
362	berry	3	Exposed	438	26.385.406	22.061.931	10.000.000
364	berry	3	Exposed	371	39.385.740	31.856.482	10.000.000
553	berry	5	Exposed	392	34.111.834	29.293.847	10.000.000
562	berry	5	Exposed	413	35.755.645	30.681.146	10.000.000
564	berry	5	Exposed	488	30.893.936	26.387.451	10.000.000
152	berry	1	Shaded	422	50.241.607	43.243.828	10.000.000
154	berry	1	Shaded	423	31.895.164	28.667.961	10.000.000
163	berry	1	Shaded	375	33.437.019	28.801.048	10.000.000
252	berry	2	Shaded	394	49.451.451	40.653.774	10.000.000
254	berry	2	Shaded	510	76.826.242	63.323.588	10.000.000
263	berry	2	Shaded	389	32.151.902	26.736.526	10.000.000
352	berry	3	Shaded	435	33.208.551	28.201.364	10.000.000
354	berry	3	Shaded	379	30.181.872	24.517.537	10.000.000
363	berry	3	Shaded	403	34.364.921	28.512.881	10.000.000
552	berry	5	Shaded	410	33.055.895	27.184.120	10.000.000
554	berry	5	Shaded	437	35.551.179	29.318.502	10.000.000
563	berry	5	Shaded	496	32.204.175	28.358.592	10.000.000
P11	Pulp	1	Shaded	473	28.769.673	25.781.061	10.000.000
P12	Pulp	1	Shaded	413	39.438.165	33.592.734	10.000.000
P13	Pulp	1	Shaded	528	27.172.832	24.047.688	10.000.000
P31	Pulp	3	Shaded	381	29.591.673	25.098.285	10.000.000
P32	Pulp	3	Shaded	476	38.544.894	31.164.065	10.000.000
P33	Pulp	3	Shaded	440	32.038.270	26.783.652	10.000.000
P51	Pulp	5	Shaded	468	40.963.864	34.200.610	10.000.000
P52	Pulp	5	Shaded	393	32.150.915	26.678.938	10.000.000
P53	Pulp	5	Shaded	418	31.575.120	26.331.872	10.000.000
TOTAL FRAGMENTS (100X2) FOR DE NOVO ASSEMBLY							330.000.000

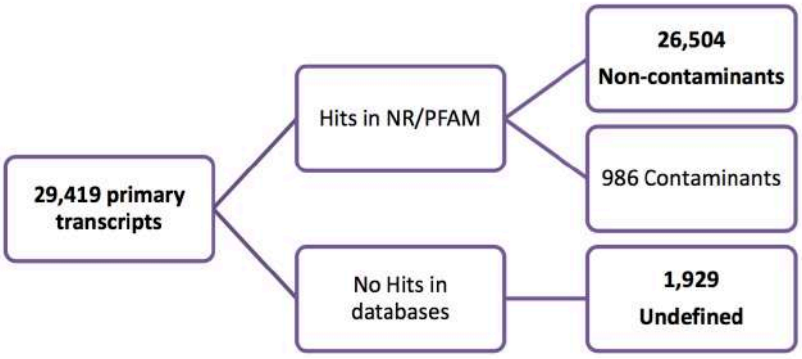

Figure A5.2. Functional characterization pipeline of the primary transcripts identified.

A5.2 Results

A5.2.1 Statistical results pertaining to the *de novo* assembly of the Sauvignon blanc transcriptome.

Table A5.2. Results of intersection of coordinates of alignment files produced with GMAP and grape V1 annotation.

	De novo transcripts
uniquely mapping	21,083
mean coverage	98.66
mean identity	98.53
multi-mapping	4,137
mean coverage	90.35
mean identity	93.47
putative chimeras	2,993
non-mapping transcripts	220

Addendum B to Chapter 5

This Addendum contains relevant and additional data not shown in Chapter 5.

B5.1 Materials and Methods

B5.1.1 The Hsf encoding genes in the grapevine genome and their expression patterns.

In order to explore and characterize the expression of Hsf encoding genes in the grapevine genome (version V1), accessions of these genes were obtained from a recent study in which they were identified (Hu et al., 2016). In the present study, the NCBI accessions for each of the nineteen Hsf genes were extracted from the abovementioned publication and their nucleotide sequences obtained with which a nucleotide BLAST was performed in the Grape Genome Database of Cribi (Centro di Ricerca Interdipratimentale per le Biotecnologie Innovative; Vitulo et al., 2014) in order to identify the gene accessions (VIT_) and the currently available functional annotation for each putative *VviHsf*.

The expression of the *VviHsfs* in various tissues and cultivars was explored by comparing data generated from previously published investigations against the RNASeq data generated for grapes (pericarp, skin and pulp separately) under control conditions in this study. For the purpose of identifying and comparing tissue-specific expression of the *VviHsfs*, microarray data generated form the grapevine gene expression atlas was utilized (Fasoli et al., 2012). Similarly, in order to explore the cultivar-specific expression patterns of the nineteen *VviHsfs*, RNASeq data generated in this investigated was compared to the expression of these genes in 5 red and 5 white Italian cultivars as generated by RNASeq analysis (Massonnet et al., 2017). The expression values of the Hsf encoding genes were normalized separately by dividing each expression value with the average expression value calculated for all the *VviHsfs* within each experiment, respectively. Hereby, the ratio of expression within each experiment could be effectively compared between different experiments by taking the inherent differences between the experimental methods and/or practices into account. The constitutive expression between various grapevine tissues and cultivars were represented in the form of heat-maps generated in the Multi-Experiment Viewer (MeV; Saeed et al., 2006).

A nucleotide BLAST was performed for each of the *VviHsf* cDNA sequences generated by *de novo* transcriptome assembly for Sauvignon blanc in Cribi (Vitulo et al., 2014) in order to identify the level of identity between these gene sequences represented in the Sauvignon blanc transcriptome and Pinot noir reference genome (PN40024).

The expression of the Abscisic acid responsive element protein (AREBs) encoding genes were further targeted in the differential expression results of the RNASeq data comparing exposed to control grapes at four developmental stages.

B5.2 Results

B5.2.1 Expression patterns of *VviHsfs*

B5.2.1.1 Tissue-specific expression of putative *Hsf* genes in grapevine

In order to determine the tissue-specific expression patterns of the nineteen *VviHsfs*, these genes were targeted in the grapevine (cv. Corvina) gene expression atlas data (Figure B5.1A; Fasoli et al., 2012). Among the Hsfs that showed consistent constitutive expression in all grapevine tissues investigated were *VviHsfA1a*, *VviHsfA1b* and *VviHsfA4a* that form part of the class a Hsfs, as well as *VviHsfB1a* that forms part of class b. Furthermore, one gene, *VviHsfB4a,* showed negligibly low expression in all grapevine tissues under non-stressed conditions. A subset of *VviHsfs* showed strong developmentally driven expression. Examples of these included the expression of *VviHsfA2a* that was limited to green, vegetative tissues such as green grape berries until véraison whereas the expression of *VviHsfA9a* was only prevalent in mature woody stems, dormant buds and mature seeds.

To further focus on Hsf expression in grape berries in particular, the expression of these nineteen Hsfs were compared between the Sauvignon blanc data generated in this investigation and the data published in the grapevine expression atlas (Figure 1B). These results revealed that *VviHsfA1a*, *VviHsfA1b* and *VviHsfB1a* were constitutively expressed in all Sauvignon blanc grape berry tissues throughout development, similar to what was reported in the grapevine gene atlas (Fasoli et al., 2012). Moreover, most of the genes that showed constitutive expression in the published data were similarly expressed in the transcriptome of Sauvignon blanc. These include *VviHsfA9a* that showed no expression in any grape berry tissues whatsoever and *VviHsfA2a* that showed developmental regulation by only being constitutively expressed during the early berry developmental stages until véraison in both experiments. Furthermore, *VviHsfA6b, VviHsfA9a, VviHsfB3a, VviHsfB4a* and *VviHsfB4b* were not expressed in any of the grape tissues evaluated. Although most of these *VviHsfs* display similar expression patterns when comparing these two datasets, some distinct differences did emerge. These include higher expression levels of *VviHsfA4a* and *VviHsfA4b* throughout development in the gene atlas data, as well as notably higher levels of expression in *VviHsfC1a* Sauvignon blanc berries.

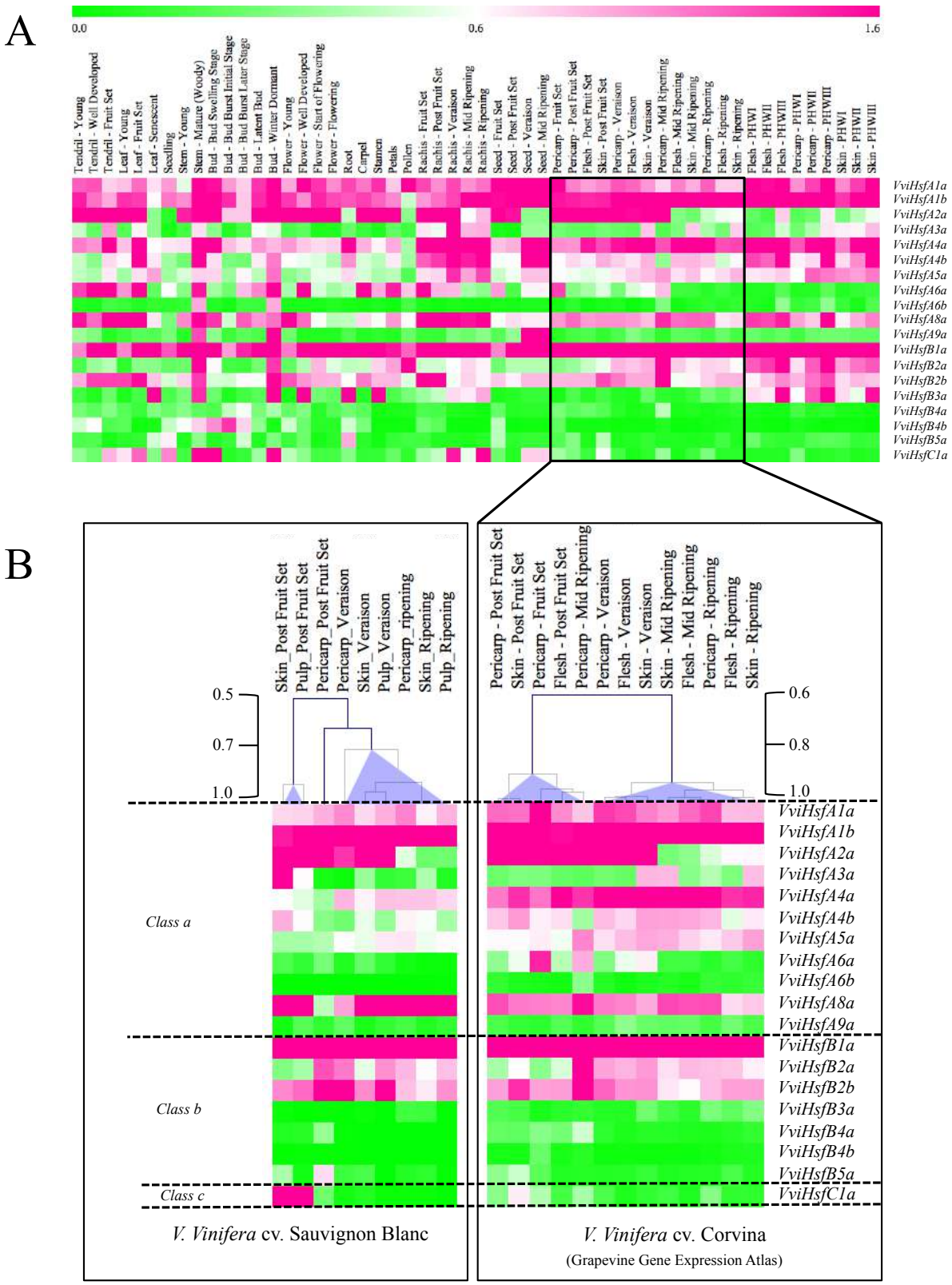

Figure B5.3. Heat maps depicting the tissue-specific expression of the 19 putative *VviHsfs* in various grapevine tissues. A: Heat map depicting the relative* expression of the *VviHsfs* as generated by the grapevine gene expression atlas (Fasoli et al., 2012). B: Heat maps comparing the expression of *VviHsfs* in Sauvignon blanc and Corvina grape berries at various developmental stages under control conditions.* refers to the expression ratio relative to each separate experiment (described in materials and methods).

B5.2.1.2 Cultivar specific expression patterns of Hsfs in grapes from various *V. vinifera* genotypes

To further broaden our investigation into how Hsf expression may differ between grapevine genotypes, the expression of these genes in whole Sauvignon berries under control conditions (Figure B5.2A) were compared to their expression in the whole berry samples of ten Italian grape cultivars during the same developmental stages (Figure B5.2B). These ten cultivars represented five red and five white wine grape varieties (Massonnet et al., 2017). Taken together, the expression patterns of these nineteen *VviHsfs* were remarkably similar between all cultivars investigated. The grape samples were clearly clustered according to the main developmental phases with green and pre-véraison grapes forming one large cluster against the grapes sampled at véraison and harvest (ripe).

Among all the genes investigated, *VviHsfA4a*, *VviHsfA4b* and *VviHsfA5a* appeared to be expressed at lower levels in Sauvignon blanc grapes during the earlier developmental stages compared to the other cultivars whereas *VviHsfA2a* and *VviHsfB2a*, showed slightly higher levels of expression at specific distinct stages in Sauvignon blanc. Furthermore, *VviHsfA1a* was expressed at higher levels in three of the red cultivars when the berries were ripe, compared to any of the other cultivars investigated (Figure B5.2).

When interpreting grapevine transcriptional data generated by means of RNASeq analysis as presented in Figure B5.2, it is important to consider that regardless of the genotypic source of the mRNA used to perform the analysis with, the sequence reads are ultimately aligned to the *V. vinifera* Pinot noir reference genome (PN40024). Unique cultivar/genotype-specific gene expression nuances may be lost as a result of the alignment of cultivars that are potentially highly divergent form the Pinot noir genome.

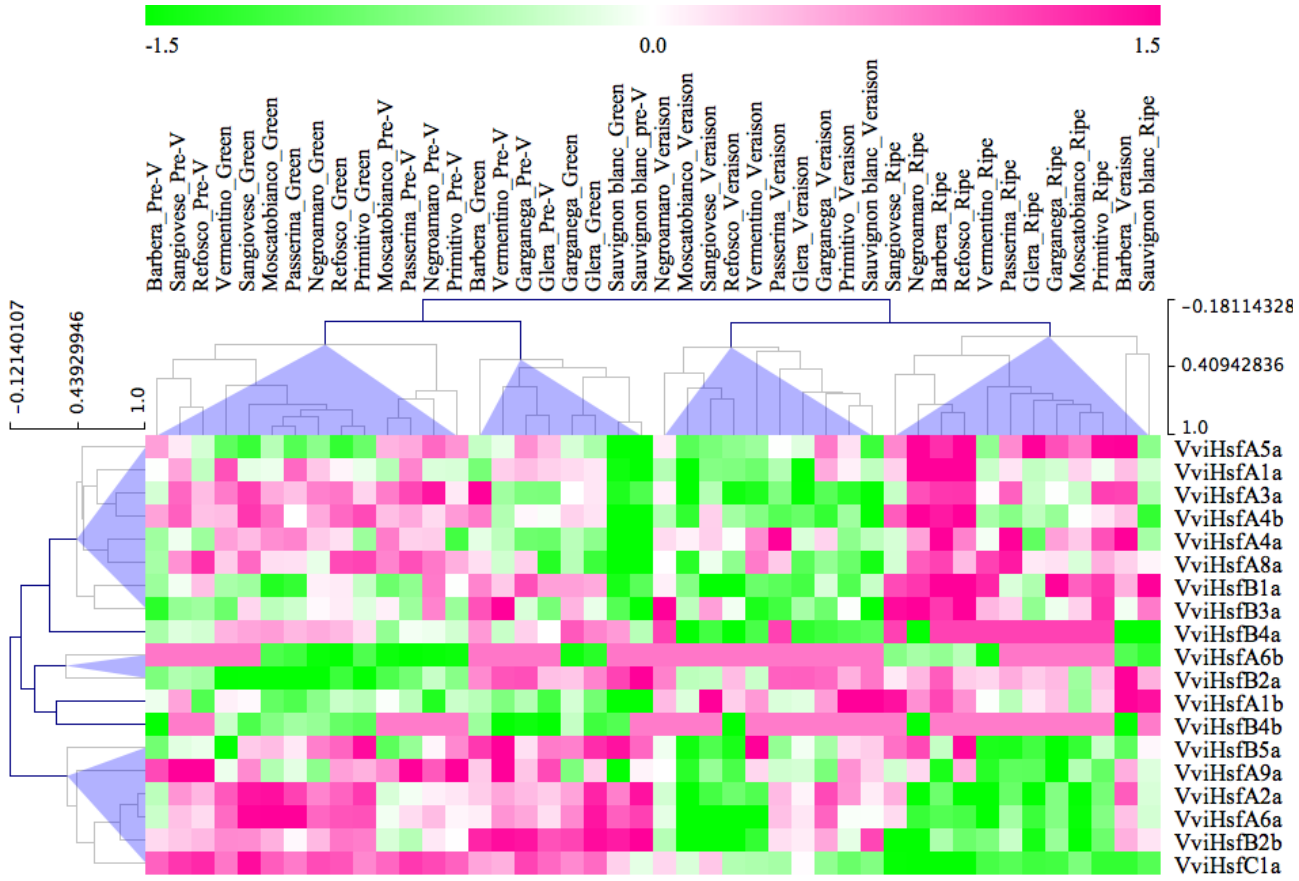

Figure B5.4. Hierarchical clustering analysis results depicting the relative* expression of each of the 19 *VviHsfs* in eleven *V. vinifera* berry genotypes.* Refers to the expression ratio relative to each separate experiment (described in materials and methods).

B5.3 References

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6879(06)11009-5.

Vitulo, N., Forcato, C., Carpinelli, E. C., Telatin, A., Campagna, D., D'Angelo, M., & Valle, G. (2014). A deep survey of alternative splicing in grape reveals changes in the splicing machinery related to tissue, stress condition and genotype. *BMC Plant Biology*. 14, 99. http://doi.org/10.1186/1471-2229-14-99.

Addendum C to Chapter 5

This Addendum contains relevant and additional data not shown in Chapter 5.

C5.1 The effect of light on the expression of Absicisic acid responsive binding protein genes.

Table C5.1. Significance of the differential expression of the Abscisic acid responsive element binding proteins (AREB) encoding genes in *V. vinifera* at four developmental stages. Numbers indicate the *q*-value when comparing exposed to control grapes at each developmental stage.

Gene accession	Funtional annotation	Green	Pre-Véraison	Véraison	Ripe
VIT_06s0009g01790	ABA-responsive element-binding protein 3 (AREB3)	0.94	0.50	0.15	0.64
VIT_13s0175g00120	ABA-responsive element-binding protein 3 (AREB3)	0.98	0.99	0.89	0.09
VIT_19s0015g01020	ABA-responsive element-binding protein ABF2	0.51	0.20	0.60	0.46
VIT_03s0063g00310	ABA-responsive element-binding protein ABF4	0.75	0.79	***0.03***	0.87

C5.2 The genes putatively co-expressed with specific VviHsfs

Table C5.2. Accessions and functional annotations of the genes similarly and uniquely putatively co-expressed with *VviHsfA2a, VviHsfA6a* and *VviHsfB2a*.

Gene accession	Functional annotation (12X)
Shared between: *VviHsfA2a VviHsfA6a VviHsfB2a*	
VIT_02s0154g00040	Thylakoid lumenal protein
VIT_08s0007g06710	Aha1 domain-containing protein
VIT_09s0070g00060	CCR4-NOT transcription complex protein
Shared between: *VviHsfA2a VviHsfA6a*	
VIT_14s0108g01500	APG6/CLPB-P/CLPB3
VIT_19s0014g03500	Unknown protein
VIT_04s0008g05870	CLPB-M/CLPB4/HSP98.7
VIT_17s0000g07190	Heat shock protein 101
VIT_04s0023g01240	Anthocyanidin 3-O-glucosyltransferase
VIT_18s0041g01230	Heat shock protein 70
VIT_16s0050g01150	heat shock protein 81-1
VIT_02s0154g00520	aspartyl protease
VIT_16s0050g02460	beta-1,3-galactosyltransferase sqv-2
VIT_12s0059g01150	dehydrogenase
VIT_13s0019g03000	heat shock protein 18.5 kDa class I
VIT_01s0011g02510	zinc finger (DNL type)
VIT_13s0047g00110	Ripening regulated protein DDTFR8
VIT_12s0034g00060	Flavonoid-glucosyltransferase 6 (Fragment)
VIT_08s0007g07380	molecular chaperone DnaJ
VIT_01s0011g00880	uvrB/uvrC motif-containing protein
VIT_13s0064g00900	Unknown protein

VIT_13s0019g02770	heat shock protein 16.9 kDa class I
VIT_03s0091g00230	Unknown protein
VIT_01s0010g01450	Unknown protein
VIT_16s0022g00470	peroxisomal biogenesis factor 11 (PEX11)
VIT_08s0040g01380	acylphosphatase family
VIT_16s0050g00750	short-chain dehydrogenase/reductase
VIT_09s0070g00380	Unknown protein
VIT_01s0011g04990	chaperonin
VIT_19s0085g01050	heat shock protein HSP17
VIT_13s0019g02740	heat shock protein 16.9 kDa class I
VIT_14s0006g00630	Dehydroascorbate reductase
VIT_04s0043g00310	ribulose-phosphate 3-epimerase
VIT_17s0000g04020	ATP-dependent Clp protease adaptor protein ClpS containing protein
VIT_13s0047g00910	molybdopterin biosynthesis MoaE
VIT_04s0008g01510	Heat shock protein (HSP17.6-CII)
VIT_19s0090g01750	Unknown protein
VIT_01s0010g02290	heat shock protein 26a, chloroplast
VIT_13s0019g03090	Heat-shock protein Low molecular weight
VIT_06s0004g01220	PAP/fibrillin family
VIT_08s0007g00060	5-oxoprolinase
VIT_08s0007g00920	Tropinone reductases
VIT_04s0008g01490	heat shock protein Cytosolic class II low molecular weight
VIT_13s0019g03170	Heat-shock protein 18.6 kDa
VIT_03s0088g00400	tubulin alpha-6 chain
VIT_06s0061g00270	Ribulose BisCO large subunit-binding protein subunit alpha, chloroplast precursor
VIT_13s0019g02930	heat shock protein 17.4 kDa class I
VIT_15s0021g00830	Chaperonin 10, Chloroplast
VIT_00s0187g00020	no hit
VIT_13s0067g03470	Glutathione S-transferase GSTO1
VIT_18s0157g00200	Steroleosin-B
VIT_17s0000g04680	signal peptide peptidase SPPA
VIT_06s0004g00240	chaperonin
VIT_17s0000g09550	CYP71A26
VIT_04s0008g04740	GCN5 N-acetyltransferase (GNAT)
VIT_12s0057g00670	heat shock protein 83
VIT_11s0016g04080	Multiprotein-bridging factor 1c MBF1C
VIT_11s0016g00650	Chaperonin 21, Chloroplast
VIT_13s0019g02780	Heat shock protein Hsp20
VIT_00s0189g00050	no hit
VIT_11s0052g00680	Unknown protein
VIT_17s0000g04550	cupin, RmlC-type
VIT_13s0019g05250	Malate dehydrogenase [NADP], chloroplast precursor (NADP-MDH)
VIT_18s0001g05220	WD-40 repeat

Shared between: *VviHsfA2a VviHsfB2a*

VIT_17s0000g08950	D111/G-patch
VIT_08s0007g03200	RNA-binding region RNP-1
VIT_07s0005g03070	LHW (LONESOME HIGHWAY)

VIT_00s1274g00010	phosphosulfolactate synthase protein
VIT_17s0000g04520	Cell growth defect factor 1
VIT_04s0008g04480	zinc finger (C3HC4-type RING finger)
VIT_14s0066g02630	Pre-mRNA cleavage complex II protein Clp1
VIT_08s0007g00740	armadillo/beta-catenin repeat
VIT_16s0022g00510	Heat shock 22 kDa protein
VIT_06s0004g04470	heat shock cognate 70 kDa protein 1
VIT_18s0001g07120	transport inhibitor response 1 protein
VIT_18s0001g13060	C3H2C3 RING-finger protein
Unique to *VviHsfA2a*	
VIT_14s0066g01210	carbonic anhydrase, chloroplast precursor
VIT_07s0129g00470	Unknown protein
VIT_01s0010g03660	Unknown protein
VIT_13s0019g04360	GCN5 N-acetyltransferase (GNAT)
VIT_16s0100g00640	rhomboid ATRBL2
VIT_09s0054g01750	Unknown protein
VIT_05s0029g00850	TIR-NBS-TIR type disease resistance protein
VIT_19s0093g00510	S-2-hydroxy-acid oxidase, peroxisomal
VIT_04s0044g01090	FAS2 (FASCIATA 2)
VIT_01s0011g03690	ER6 protein universal stress protein (USP) family
VIT_18s0001g08500	lipase family
VIT_11s0052g00040	macrophage migration inhibitory factor
VIT_02s0025g04060	Rab/Ypt GTPase Ara4-interacting protein
VIT_04s0008g06850	biopterin transport-related protein BT1
VIT_07s0005g01980	glycosyl transferase family 8 protein
VIT_06s0080g01110	transcription factor (E2F) E2F1
VIT_14s0030g01580	AIN1 (ACC INSENSITIVE 1) 5'-3' exoribonuclease (XRN4)
VIT_16s0098g01240	Unknown protein
VIT_01s0011g01380	THIOREDOXIN O1 ATO1
VIT_06s0004g06540	undecaprenyl pyrophosphate synthetase
VIT_09s0070g00820	AAA-type ATPase
VIT_05s0049g01360	HrBP1-1 PAP/fibrillin family
VIT_04s0008g02280	Unknown protein
VIT_06s0004g05770	heat shock protein 17.4 kDa class I
VIT_01s0011g04410	APG2 (ALBINO AND PALE GREEN 2)
VIT_07s0005g01970	galactinol synthase
VIT_13s0084g00080	MAP65/ASE1; t-snare
VIT_00s0194g00030	heat shock protein 81-4
VIT_07s0031g02510	sirtuin (silent mating type information regulation 2 homolog)
VIT_10s0003g05470	FAD-binding domain-containing protein
VIT_11s0016g00850	RecQl3 (Recq 3); ATP binding / ATP-dependent helicase
VIT_18s0001g11980	NAC domain containing protein 57
VIT_03s0063g02060	transducin family protein / WD-40 repeat
VIT_08s0007g00840	ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor
VIT_03s0088g00100	Concanavalin A lectin
VIT_15s0021g01420	nuclear RNA-binding protein
VIT_18s0001g10620	no hit
VIT_09s0002g00690	DnaJ homolog, subfamily B, member 6

VIT_07s0005g00610	tubulin gamma complex component 4
VIT_16s0100g00410	TraB protein
VIT_01s0011g01090	Unknown protein
VIT_01s0150g00060	SOUL heme-binding
VIT_01s0011g03700	acyl-CoA thioesterase
VIT_09s0002g07840	NADPH quinone oxidoreductase-like protein
VIT_18s0122g00190	Unknown protein
VIT_17s0000g08200	phosphoglycerate/bisphosphoglycerate mutase
VIT_01s0011g05710	HEAT repeat-containing protein
VIT_03s0017g01370	UDP-glycosyltransferase 85A8
VIT_05s0077g01410	no hit
VIT_00s0179g00150	heat shock transcription factor A6B
VIT_14s0081g00710	unknown protein
VIT_03s0091g00850	Unknown protein
VIT_18s0001g13520	zinc finger (B-box type)
VIT_07s0005g00680	exocyst complex subunit Sec15B
VIT_13s0019g02760	heat shock protein 16.9 kDa class I
VIT_18s0001g06570	no hit
VIT_16s0050g01260	Unknown protein
VIT_11s0206g00130	VAMP-like protein YKT61
VIT_05s0062g00830	Ribosomal protein S1
VIT_12s0028g01230	fanconi anemia, complementation group D2
VIT_01s0026g02480	CP12-2
VIT_14s0036g00630	RNA binding motif protein 42
VIT_13s0019g02840	heat shock protein
VIT_15s0021g00320	no hit
VIT_00s0225g00130	Alanine transaminase.
VIT_11s0206g00120	lipocalin, Temperature-induced
VIT_19s0015g02240	DYNAMIN-LIKE 3
VIT_09s0002g08260	PRLI-interacting factor L
VIT_12s0028g01240	fanconi anemia, complementation group D2
VIT_14s0108g01110	Cystathionine gamma-synthase isoform 2
VIT_00s1286g00010	heat shock protein STI
VIT_18s0001g10130	zinc finger (C2H2 type) family
VIT_14s0068g02020	yrdC
VIT_16s0050g01030	no hit
VIT_18s0001g10630	no hit
VIT_05s0051g00340	Chaperonin CPN60-2 (HSP60-2)
VIT_01s0011g00850	Unknown protein
VIT_10s0003g03090	3-beta hydroxysteroid dehydrogenase
VIT_13s0084g00660	basic/Leu zipper protein HBP-1b(c1)
VIT_17s0000g03920	octicosapeptide/Phox/Bem1p (PB1) domain-containing protein
VIT_13s0019g02850	heat shock protein 17.4 kDa class I
VIT_00s0187g00040	Cystatin
VIT_18s0089g01210	Cupin super
VIT_17s0000g08170	chaperonin
VIT_02s0025g04640	Unknown protein
VIT_04s0008g05210	bZIP protein HY5 (HY5)

VIT_12s0028g01740	DNAJ heat shock N-terminal domain-containing protein
VIT_07s0005g01800	Agenet domain-containing protein
VIT_18s0001g10610	no hit
VIT_12s0028g03790	no hit
VIT_07s0005g03440	structural maintenance of chromosomes (SMC1)
VIT_01s0011g06440	chalcone reductase
VIT_13s0019g01060	SC35 splicing factor, 30 kD (SCL30)
VIT_07s0031g00680	red chlorophyll catabolite reductase (accelerated cell death 2)
VIT_05s0020g00480	mitochondrial substrate carrier family protein
VIT_06s0009g01990	Anthocyanin 3-O-galactosyltransferase
VIT_09s0002g05840	RPS5 (RESISTANT TO P. SYRINGAE 5)
VIT_10s0116g01550	Unknown protein
VIT_06s0004g07690	pentatricopeptide (PPR) repeat-containing
VIT_02s0025g04180	Unknown protein
VIT_05s0077g01880	DAG protein
VIT_15s0021g00140	speckle-type POZ protein-related
VIT_13s0019g02820	heat shock protein
VIT_07s0031g01730	iron-sulfur assembly protein IscA, chloroplast precursor
VIT_04s0008g01590	heat shock protein Cytosolic class II low molecular weight
VIT_01s0011g06310	Inositol polyphosphate related phosphatase

Unique to *VviHsfA6a*

VIT_14s0006g01890	Unknown protein
VIT_19s0090g00910	alanine racemase
VIT_03s0038g02670	Unknown protein
VIT_07s0005g00030	GST2
VIT_17s0000g04690	protease SppA
VIT_14s0083g01020	regulator of chromosome condensation (RCC1)
VIT_00s0211g00120	Glycine hydroxymethyltransferase
VIT_18s0001g00070	pentatricopeptide (PPR) repeat-containing protein
VIT_13s0074g00790	CYP94C9
VIT_01s0026g00670	Unknown protein
VIT_07s0151g01000	Photosystem I reaction center subunit II (PSAD)
VIT_00s0684g00030	SEN1 (DARK INDUCIBLE 1)
VIT_07s0005g03680	Non-intrinsic ABC protein 4
VIT_00s0958g00010	Coatamer alpha subunit (HEPCOP)
VIT_12s0034g00040	UDP-glucose glucosyltransferase
VIT_14s0006g01290	myb domain protein 113
VIT_01s0011g02150	photosystem II stability/assembly factor, chloroplast (HCF136)
VIT_08s0040g03000	no hit
VIT_19s0014g00080	steroid 5alpha-reductase
VIT_08s0007g05010	Unknown protein
VIT_01s0011g03010	serine/threonine-protein kinase SNT7, chloroplast precursor
VIT_05s0094g00140	no hit
VIT_02s0025g05100	AT-hook DNA-binding protein
VIT_15s0024g00480	ATPP2-A2
VIT_04s0008g01110	heat shock transcription factor A6B
VIT_07s0031g01560	Unknown protein
VIT_05s0102g00600	no hit

VIT_16s0013g01640	F-type H+-transporting ATPase epsilon chain
VIT_02s0025g02640	Unknown protein
VIT_18s0041g00280	T-complex protein 11
VIT_12s0055g00900	RAE1 RNA export 1 homolog
VIT_13s0019g03160	heat shock protein 18.1 kDa class I
VIT_00s0323g00030	HSP associated protein
VIT_13s0067g02460	SAC3/GANP
VIT_08s0007g04000	Unknown protein
VIT_11s0016g02350	ubiquinone/menaquinone biosynthesis methyltransferase UbiE
VIT_00s0317g00050	dehydroascorbate reductase
VIT_04s0008g01570	heat shock protein Cytosolic class II low molecular weight
VIT_16s0013g00920	no hit
VIT_07s0151g00280	Bax inhibitor
VIT_19s0015g01100	peptidyl-prolyl cis-trans isomerase, FKBP-type
VIT_08s0007g08880	no hit
VIT_01s0127g00740	quinone oxidoreductase, chloroplast precursor
VIT_03s0088g00320	Peptidase M50
VIT_18s0001g11150	acyl-peptide hydrolase
VIT_06s0080g00280	bile acid sodium symporter
VIT_11s0037g01270	disease resistance protein (NBS-LRR class)
VIT_13s0019g00870	Unknown protein
VIT_04s0023g00720	Unknown protein
VIT_18s0001g05440	Methyltransferase type 11
VIT_09s0002g08460	8-amino-7-oxononanoate synthase
VIT_08s0007g03370	Unknown protein
VIT_05s0049g01080	Glutathione S-transferase 25 GSTU7
VIT_14s0030g01650	Unknown protein
VIT_10s0092g00050	ribosomal protein L17
VIT_15s0024g01170	no hit
VIT_19s0027g00130	translation initiation factor IF-2, chloroplast
VIT_08s0056g01690	LrgB-like family protein
VIT_18s0001g02740	photosystem II 22 kDa protein PSBS
VIT_16s0050g01670	UDP-glucose:isoflavone 7-O-glucosyltransferase
VIT_08s0040g00790	FK506-binding protein 4/5
VIT_06s0004g08200	ubiquitin-conjugating enzyme E2 D/E
VIT_13s0019g01030	binding
VIT_07s0031g01890	no hit
VIT_18s0001g10460	defective chloroplasts and leaves protein / DCL protein
VIT_00s0960g00010	phosphosulfolactate synthase protein
VIT_07s0005g01610	Unknown protein
VIT_18s0001g01430	oxidoreductase N-terminal domain-containing
VIT_13s0067g00500	transcription elongation factor SPT6
VIT_02s0025g00280	heat shock protein 81-1
VIT_03s0063g01310	oxidoreductase, 2OG-Fe(II) oxygenase family
VIT_08s0040g01470	cytokinin-O-glucosyltransferase 2
VIT_06s0080g00980	secoisolariciresinol dehydrogenase
VIT_05s0049g00300	1-aminocyclopropane-1-carboxylate oxidase homolog 1
VIT_18s0001g01660	NADH dehydrogenase I subunit M

VIT_12s0055g00170	UDP-glucose glucosyltransferase
VIT_03s0038g03250	vestitone reductase
VIT_18s0001g10640	no hit
VIT_18s0122g00960	glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor
VIT_12s0035g01080	carotenoid isomerase 1, chloroplast precursor
VIT_05s0049g01620	cyclopropane fatty acid synthase
VIT_08s0007g03270	SOUL heme-binding protein
VIT_10s0042g00200	thioredoxin X
Unique to *VviHsfB2a*	
VIT_09s0002g00640	Small heat stress protein class CIII
VIT_02s0154g00480	heat shock protein MTSHP
VIT_01s0146g00150	BCL-2-ASSOCIATED ATHANOGENE 5
VIT_14s0068g01890	Nodulin
VIT_10s0003g00260	DnaJ homolog, subfamily B, member 4
VIT_04s0008g03070	no hit
VIT_16s0022g02140	CYP704A2
VIT_05s0020g02450	FAR1-RELATED SEQUENCE 7
VIT_07s0185g00040	Unknown protein
VIT_12s0035g00740	no hit
VIT_09s0002g00630	no hit
VIT_07s0151g00660	Ribosomal protein L24 (At5g23535) 50S
VIT_00s0188g00110	glucose-inhibited division family A
VIT_09s0002g09040	patellin-5
VIT_11s0078g00260	Unknown protein
VIT_18s0076g00370	ribosomal protein P2 (RPP2A) acidic 60S
VIT_14s0066g00540	EMB3012 (EMBRYO DEFECTIVE 3012)
VIT_16s0050g01140	glutamine cyclotransferase
VIT_17s0000g02320	glutaredoxin
VIT_14s0006g02440	germin-like protein 3 [Vitis vinifera]
VIT_05s0020g03780	embryo sac development arrest 15
VIT_01s0026g02540	zinc finger (C3HC4-type RING finger)
VIT_13s0139g00290	Disease resistance protein

Chapter 6

The transcriptional effect of elevated light exposure on the metabolic pathways associated with methoxypyrazine and volatile thiol synthesis in Sauvignon Blanc grapes.

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The transcriptional effect of elevated light exposure on the metabolic pathways associated with methoxypyrazine and volatile thiol synthesis in Sauvignon Blanc grapes.

6.1 Introduction

Sauvignon Blanc wines are typically categorized according to "tropical" and/or "green" aroma characters. The tropical aromas are associated with specific descriptors for passion fruit, guava, grapefruit and gooseberry, whereas the green aromas include those of capsicum, tomato leaf and asparagus among others. The compounds known to be attributed to each of these flavor classes have been well studied in Sauvignon Blanc wines - volatile thiols are predominantly responsible for the perception of the tropical characteristics, whereas methoxypyrazines are frequently, although not exclusively, responsible for the green characteristics in these wines (for review see Coetzee et al., 2012).

Among the most important volatile thiols present in Sauvignon Blanc wines are various combinations of 3-sulfanylhexan-1-ol (3SH, formerly known as 3MH) responsible for the grapefruit and passion fruit aromas (Tominaga et al., 1998), its acetate 3-sulfanylhexyl acetate (3SHA formerly known as 3MHA) and 4-methyl-4-sulfanylpentan-2-one (4SMP, formerly known as 4MMP) responsible for the box tree and blackcurrant aromas (Tominanga et al., 1998). The presence of 3SH and 4MSP are virtually undetectable in grape berries and juices in the free-form (Capone et al., 2011), but their non-volatile precursors are synthesized from the metabolism of green leaf volatiles (GLVs) after which they are liberated and volatilized by yeast enzymes during alcoholic fermentation (Kobayashi et al., 2011; Swiegers and Pretorius, 2007; Tominaga et al., 1998).

The synthesis of these thiol precursors are generally initiated by the activity of several lipoxygenase enzymes (LOXs) responsible for the degradation of polyunsaturated fatty acids (PUFA) that mostly include linoleic and linolenic acids into PUFA hydroperoxides in grape berries (Podolyan et al., 2010; Qian et al., 2015). These hydroperoxides are rapidly converted into a wide range of down-stream compounds collectively called oxylipins. Oxylipins are enzymatically synthesized by several members of the Cytochrome P450 (CYP74) enzyme family that include hydroperoxide lyase (HPL), allene oxide synthase (AOS), divinyl ether synthase (DES) and epoxy alcohol synthase (EAS) that are responsible for the synthesis of C6-volatiles, jasmonates, divinyl ether PUFAs and epoxy hydroxyl PUFAs, respectively (Podolyan et al., 2010). Several LOXs additionally have the ability to act on PUFA hydroperoxides themselves to form keto PUFAs (Podolyan et al.,

2010). In the synthesis of thiol precursors, however, the activity of HPLs and alcohol dehydrogenases (ADHs) are responsible for the synthesis of C6-volatiles (Podolyan et al., 2010; Qian et al., 2015) are of particular interest since these green leaf volatiles (GLVs), along with methoxypyrazines, contribute to the characteristic aroma of freshly cut grass (Matsui, 2006) and further influence the style of Sauvginon Blanc wines (Benkwitz et al., 2012).

Methoxypyrazines are responsible for the vegetative, green characteristics of wine made from only a limited number of cultivars that include Cabernet Sauvignon, its parents, Sauvignon Blanc and Cabernet Franc, as well as Merlot and Carménère (Belancic and Agosin, 2007; Bowers and Meredith, 1997; Hashizume et al., 2001). Although three methoxypyrazines are readily detected in grape berries, 3-isobutyl-2-methoxypyrazine (IBMP) is detected at the highest concentrations and is synthesized during the early berry developmental stages after which it is degraded and volatilized during berry ripening. Previous studies have proposed two possible pathways involved in IBMP synthesis. The first involves the reaction between the amino acid, leucine, and glyoxyl in the formation of the precursor, 3-isobutyl-2-hydroxypyrazine (IBHP)(Murray et al., 1970), whereas the other pathway involves the reaction between leucine and glycine to form the same precursor (Cheng et al., 1991). It is, however, well established that a group of specific methyltransferase enzymes (OMTs) are responsible for the methylation of IBHP in the final step of IBMP synthesis in grapes (Dunlevy et al., 2013).

Viticultural practices have further been shown to have a significant impact on the accumulation of aroma precursor in grapes. Elevated light exposure by means of viticultural canopy manipulations have been implicated in the accumulation of more tropical aromas vs. green aromas in wines (for review, see Reynolds et al., 2010) while mechanical damage caused by machine harvesting has been associated with higher levels of thiols in wines (Olejar et al., 2015). Recent studies have confirmed the effect of leaf removal on the sensory perception of Sauvignon Blanc wines (Šuklje et al., 2016). Not only is it known that defoliation increases the accumulation of volatile thiols associated with tropical aromas (Šuklje et al., 2016), but the accumulation of methoxypyrazines in developing grapes appear to be highly sensitive to varying degrees of exposure (Royona et al., 2008; Šuklje et al., 2014; Koch et al., 2012).

As part of this study, we established a highly characterized vineyard in which the effect of a leaf removal treatment was mainly elevated light exposure to developing Sauvignon Blanc grapes (Chapter 3; Du Plessis et al., 2017; Young et al., 2016). The combination of transcriptomic and extensive metabolomic analyses of these grapes revealed that elevated light exposure activated several photoprotective mechanisms on a transcriptional level that had secondary metabolic consequences. These consequences involved the accumulation of elevated levels of secondary metabolites with sunscreening and/or antioxidative capabilities in the grapes that serve as grape and wine impact odorants as well. These metabolites included flavonols, terpenes and specific norisoprenoids (Chapter 3; Young et al., 2016; Addendum B to Chapter 3). Given the importance of the

methoxypyrazines, GLV's and the thiols on Sauvignon Blanc wine typicity, the pathways associated with accumulation of thiol precursors and methoxypyrazines remained to be explored to evaluate the transcriptional impact of the increased exposure. Here we report on these gene expression patterns and will discuss the links that could be drawn from these patterns, correlated with some metabolites measured in the berries. We further relate it to the analyses of the wines made from the grapes that were subjected to the increased exposure/control treatments.

6.2 Materials and Methods

6.2.1 Experimental materials and methods previously described

All methods pertaining to the experimental layout, growing conditions of the grapes, sampling strategy, RNA extractions, RNASeq and the *de novo* assembly of the Sauvginon Blanc transcriptome have been described in previous sections of this thesis (Du Plessis et al. 2017; Young et al., 2016; Chapter 3; Chapter 5).

The amino acid concentrations of each of the berry samples were previously determined and reported according to the methods described in Du Plessis et al., (2017; Chapter 3). Furthermore, the concentrations of a limited selection of C6 volatile compounds were determined according to the method described in Young et al., (2016; Addendum B to Chapter 3). The data for the following compounds were extracted and used further as context for the C6 volatile potential of the control and exposed grapes in the EL31, 33, 35 and 38 stages: *trans*-2-hexanal, n-hexanal and *cis*-2-hexenal.

6.2.2 Identification of grapevine genes associated with Sauvignon Blanc aroma compounds

In order to explore and characterize the expression of genes in the grapevine genome putatively involved in the synthesis of Sauvignon Blanc aroma compounds, genes identified in previous publications were targeted. For the purpose of exploring the synthesis of GLV compounds from the degradation of polyunsaturated fatty acids (PUFAs), the lipoxygenase genes and a subset of the CYP74 genes encoding hydroperoxide lyases that were characterized by Podolyan et al., (2010) were targeted along with the downstream alcohol dehydrogenase and alcohol acyl transferase genes involved in GLV synthesis (Qian et al., 2015). The genes putatively associated with the down-stream synthesis of the thiol precursor, 3SH, from the metabolism of the GLV compounds were obtained from a publication characterizing these metabolic steps (Kobayashi et al., 2011). Finally, the genes putatively responsible for the final metabolic steps towards the synthesis of the methoxypyrazine, 2-methoxy-3-isobutylpyrazine (IBMP) were identified in Dunlevy et al., (2013).

The NCBI accessions for each of these genes were extracted from the abovementioned publications and their nucleotide sequences obtained with which a nucleotide BLAST was performed in the Grape Genome Database of Cribi (Centro di Ricerca Interdipratimentale per le Biotecnologie Innovative; Vitulo et al., 2014) in order to identify the gene accessions (V1) and the currently available functional annotation for each gene.

6.2.3 Volatile thiol analysis of wine made from exposed and control Sauvignon Blanc grapes

In order to evaluate the effect that elevated light may have on the accumulation of volatile thiols in Sauvignon Blanc wines, data that was previously generated from small-scale wines produced from the same grapes (described in Coetzee et al., 2014) that were subjected to the transcriptome analysis and metabolite profiling, as described in this thesis, was utilized to support and contextualize the transcriptional data presented here. Grape juice was prepared from grapes harvested at EL38 from control and light exposed bunches that were pooled separately (Figure 6.1). These juices from control and exposed grape samples were divided into two fermentations, each inoculated with two separate commercial *S. cerevisiae* yeasts that included VIN7 (Anchor Yeast Biotechnologies) and Cross Evolution (Lallemand) in triplicate, respectively (Figure 6.1). All methods, including crushing and pressing of the grapes and all winemaking practices were performed at the Institute for Wine Biotechnology (Faculty of Agriculture, Stellenbosch University) according to the protocol described in Coetzee et al. (2014).

The concentrations of three volatile thiols, 4MSP, 3SH and 3SHA, were quantified for each of the four wines produced in triplicate according to the method described in Coetzee et al. (2013) adapted from the method published by Tominaga et al. (2000).

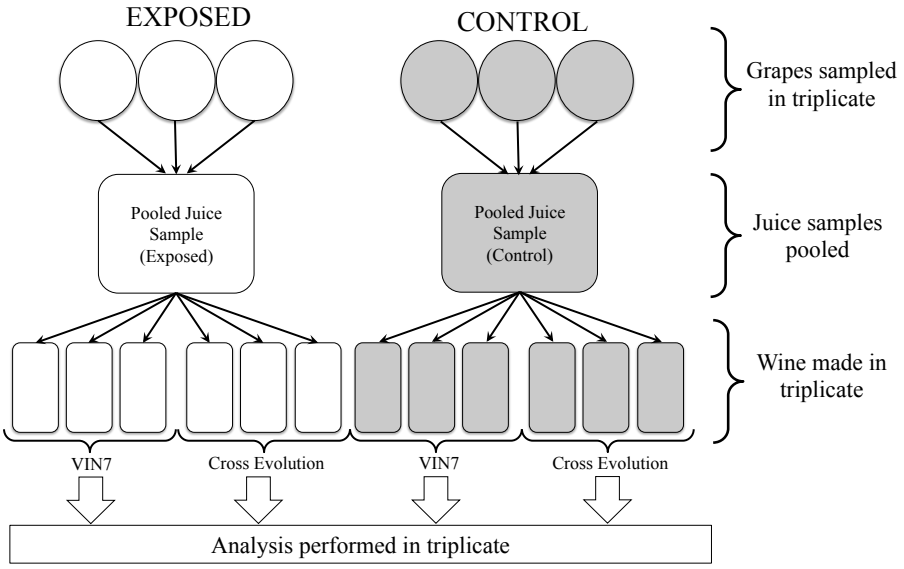

Figure 6.1. Experimental layout of the replicates included in the production of small-scale wines from exposed and control Sauvginon Blanc grapes by two commercial yeast strains (VIN7 and Cross Evolution).

6.3 Results

6.3.1 The genes involved in aroma compound synthesis in the grapevine reference genome

In total, sixteen genes previously implicated in the synthesis of Sauvignon Blanc varietal aroma compounds were identified in the grapevine genome and the accessions of these genes were targeted to further explore their expression in the RNASeq datasets generated as part of this study (Chapters 3 and 4). The accessions, the pathways in which they are putatively involved and the reference literature from which they were obtained are summarized in Table 6.1.

The expression patterns of these genes under non-stressed (control) conditions in a selection of other grape tissues and cultivars are reported in Figures A6.1 and A6.2 in Addendum A of Chapter 6. Furthermore, the level of sequence identity of these genes when comparing the Pinot noir reference genome (PN40024) and the *de novo* assembled Sauvignon Blanc transcriptome were explored and are presented in Table A6.1 and Figure A6.3 in Addendum A of Chapter 6.

Table 6.1. The genes associated with the synthesis of Sauvignon Blanc varietal aroma compounds, their accessions, annotations and the literature source of the original characterization of these genes in the respective metabolic pathways.

Pathway involved	Gene accession	Gene annotation	Reference
Lipoxygenase pathway	Lipoxygenase *(VviLOXC)*	*VIT_14s0128g00780*	Podolyan et al., 2010
	Lipoxygenase *(VviLOXO)*	*VIT_09s0002g01080*	Podolyan et al., 2010
	Lipoxygenase *(VviLOXA)*	*VIT_06s0004g01510*	Podolyan et al., 2010
	Lipoxygenase *(VviLOXL)*	*VIT_05s0020g03170*	Podolyan et al., 2010
	Lipoxygenase *(VviLOXP)*	*VIT_01s0010g02750*	Podolyan et al., 2010
	Lipoxygenase *(VviLOXE)*	*VIT_06s0004g01450*	Podolyan et al., 2010
	Lipoxygenase *(VViLOXJ)*	*VIT_13s0064g01480*	Podolyan et al., 2010
	Hydroxyperoxide lyase *(VviHPLA)*	*VIT_12s0059g01060*	Podolyan et al., 2010
	Hydroxyperoxide lyase *(VviHPLF)*	*VIT_03s0063g01820*	Podolyan et al., 2010
	Alcohol dehydrogenase *(VviADH1)*	*VIT_18s0001g15410*	Qian et al., 2015
	Alcohol dehydrogenase *(VviADH2)*	*VIT_18s0001g15450*	Qian et al., 2015
	Alcohol dehydrogenase *(VviADH3)*	*VIT_04s0044g01120*	Qian et al., 2015
	Alcohol acyl transferase *(VviAAT)*	*VIT_00s0187g00260*	Qian et al., 2015
3MH-*S*-cys synthesis pathway	Glutathione S-transferase *(VviGST3)*	*VIT_12s0028g00930*	Kobayashi et al., 2011
	Glutathione S-transferase *(VviGST4)*	*VIT_04s0079g00690*	Kobayashi et al., 2011
	Gamma-glutamyltranspeptidase *(VviGGT)*	*VIT_11s0016g02830*	Kobayashi et al., 2011
Methoxypyrazine pathway	Caffeic acid O-methyltransferase *(VviOMT1)*	*VIT_12s0059g01790*	Dunlevy et al., 2013
	Caffeic acid O-methyltransferase *(VviOMT2)*	*VIT_12s0059g01750*	Dunlevy et al., 2013
	Catechol O-methyltransferase *(VviOMT3)*	*VIT_03s0038g03090*	Dunlevy et al., 2013
	Catechol O-methyltransferase *(VviOMT4)*	*VIT_03s0038g03080*	Dunlevy et al., 2013

6.3.2 The effect of elevated light exposure on methoxypyrazine synthesis in developing Sauvignon Blanc grapes.

The effect that elevated light exposure may have on the expression of the O-methyltransferase genes responsible for the final step of methoxypyrazine synthesis was explored by comparing the expression levels of the four characterized *VviOMT* genes between light exposed and control berries sampled throughout development. The two possible pathways by which 3-isobutyl-2-hydroxypyrazine (IBHP) can be synthesized were considered and are shown in Figure 6.2A. The amino acid, glycine, but not leucine, from which IBHP is putatively synthesized, was significantly upregulated (q<0.05) by elevated light throughout berry development (Figure 6.2). Furthermore, the expression of three out of the four *VviOMTs* showed significantly lower expression (q<0.05) in exposed grapes during the green developmental stages. By considering the expression patterns of these *VviOMT* genes, it was clear that these genes were predominantly expressed during the early green developmental stages (EL31 and EL33) after which their expression declined to negligibly low levels by véraison (Figure 6.2B). The $\log_2$ fold change values of gene expression levels and compound concentrations when comparing exposed to control grapes reported in Figure 6.2 are summarized in Table B6.1; Addendum B to Chapter 6.

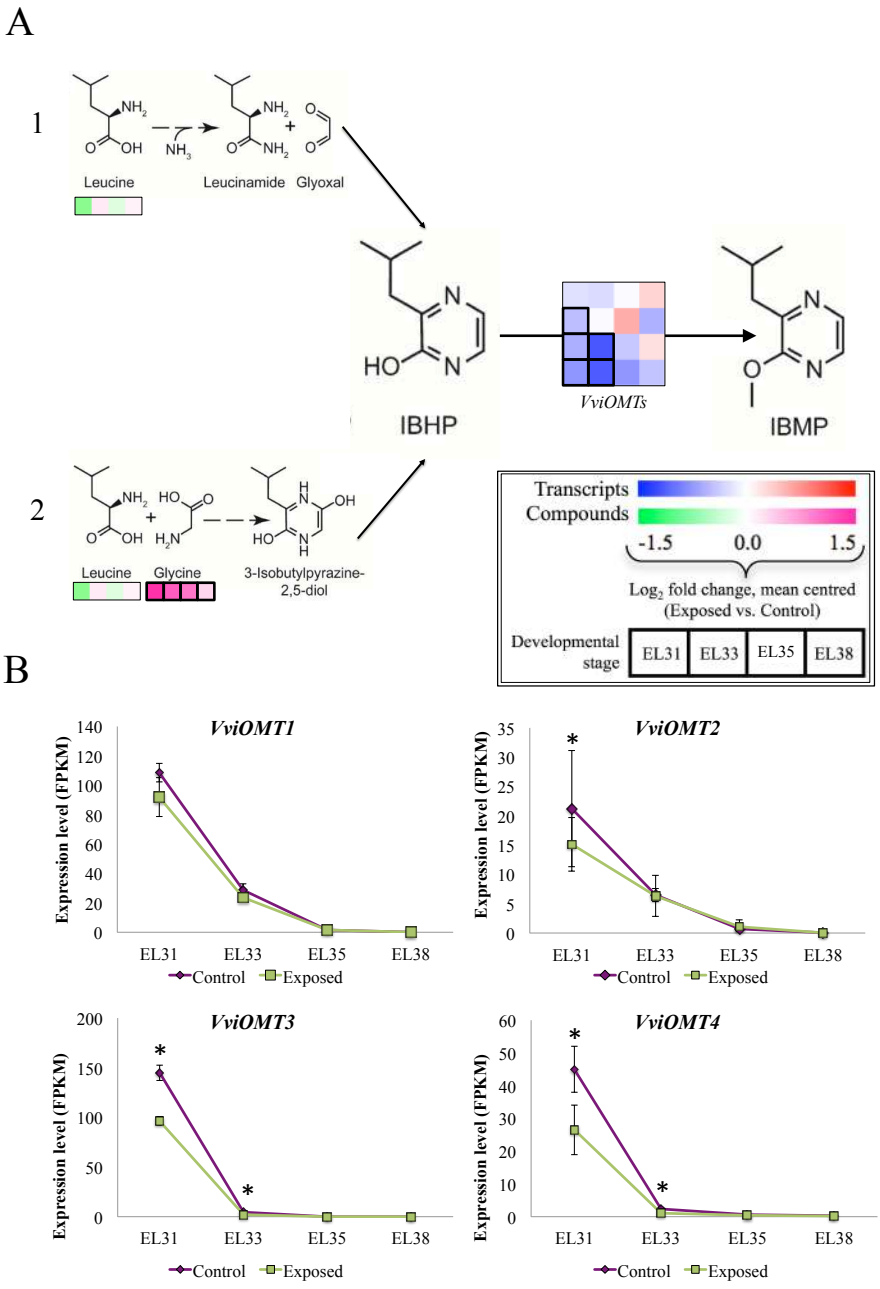

Figure 6.2. The metabolic pathways and genes associated with the synthesis of methoxypyrazines in developing grape berries adapted from Dunlevy et al., (2013). A: The two proposed pathways in the synthesis of 2-methoxy-3-isobutylpyrazine (IBMP) via the degradation of 3-isobutyl-2-hydroxypyrazine (IBHP) through the activity of *O*-methyltransferases (OMTs). Blocks indicate the mean-centered $\log_2$ fold change of the FPKM expression value of the transcripts putatively encoding the O-methyltransferase enzymes (*VviOMTs*) and the concentrations of the amino acids (mg/g fresh weight) involved in these metabolic pathways. Significant differences ($q \leq 0.05$) are indicated by a bold contour (frame). B: The expression of the four *O*-methyltransferase encoding genes at four developmental stages during berry development under control conditions and elevated light exposure. Statistical significance is indicated by an *.

6.3.3 The effect of elevated light on the synthesis of thiol precursors in developing grapes and its possible link to thiol concentrations in Sauvignon Blanc wine

In this investigation, we attempted to create an overview of some of the metabolic steps involved in the synthesis of volatile thiols in Sauvignon Blanc wines by evaluating the development of the non-volatile thiol precursors in the developing grapes on a transcriptional level. These results showed that several *VviLOX* genes, putatively involved in the degradation of polyunsaturated fatty acids (PUFAs) in grape berries were either significantly up or downregulated by elevated light exposure at various stages of berry development. During the early developmental stages, EL31 and EL33, two of these genes were significantly upregulated by elevated light and the expression of one of the CYP74 genes, *VviHPLA,* responsible for the subsequent metabolic step towards GLV synthesis was simultaneously upregulated. *VviHPLF* further showed significantly downregulated expression (q<0.05) in response to the treatment during the later developmental stages, however, it is important to note that this gene shows negligibly low expression levels (FPKM<1.0) that are statistically significant, although the biological significance could be negligible. Two of the alcohol dehydrogenase encoding genes (*VviADH1* and *VviADH3*) as well as the alcohol acyl transferase gene were further significantly upregulated by elevated light while two of the C6-volatile compounds, *trans*-2-hexanal and *cis*-2-hexenal, were further present at significantly higher concentrations (p<0.05) in exposed grapes from pre-véraison (EL33) until the grapes achieved ripeness (EL38) (Figure 6.3).

By taking a snapshot of the putatively characterized metabolic path involved in the synthesis of the precursor for one of the most important volatile thiols in Sauvignon Blanc, the genes involved in this branch of the pathway could be evaluated. None of the genes involved in the synthesis of the 3SH pro-precursor (3SH-*S*-glut), nor the *S*-cysteinylated 3SH precursor (3SH-*S*-cys) was significantly altered by elevated light exposure. Similar to the CYP74, *VviHPLF*, *VviGST4* showed significantly downregulated expression in response to the light treatment although this gene is not expressed at accurately quantifiable levels in Sauvignon Blanc. Therefore, despite the statistical significance, the biological relevance of these low expression levels needs to be established.

When the berries were ripe (EL38), the levels of GLVs were slightly elevated in exposed grapes of which wines were made using two separate *S. cerevisiae* yeasts. After wine was produced with shaded (control) and light exposed grapes, the concentrations of three of the most important volatile thiols contributing to Sauvignon Blanc varietal aroma were quantified (Figure 6.3). 4MSP were present at higher levels in wine made from exposed grapes with wine produced using the Cross Evolution commercial yeast containing significantly higher levels (p<0.05) of this volatile thiol responsible for box tree and black currant aromas. The concentration of the volatile thiol, 3SH, varied depending on the yeast with which the fermentations were performed. Exposed grapes fermented with Cross Evolution yeasts produced slightly lower concentrations of 3SH, whereas exposed grapes produced with VIN7 had significantly higher levels of 3SH (p<0.05), responsible for the aromas of passion fruit

and grapefruit. The thiol, 3SH, can also be further converted through yeast acetyltransferase activity during the winemaking process, to produce 3SHA, the volatile thiol responsible for the herbaceous box tree aroma in wines. The quantification of 3SHA in these Sauvignon Blanc wines revealed significantly lower levels ($p<0.05$) of this volatile thiol in exposed grapes regardless of the yeast strain used for fermentation purposes (Figure 6.3). The $\log_2$ fold change values of gene expression levels and compound concentrations when comparing exposed to control grapes reported in Figure 6.3 are summarized in Table B6.2; Addendum B to Chapter 6.

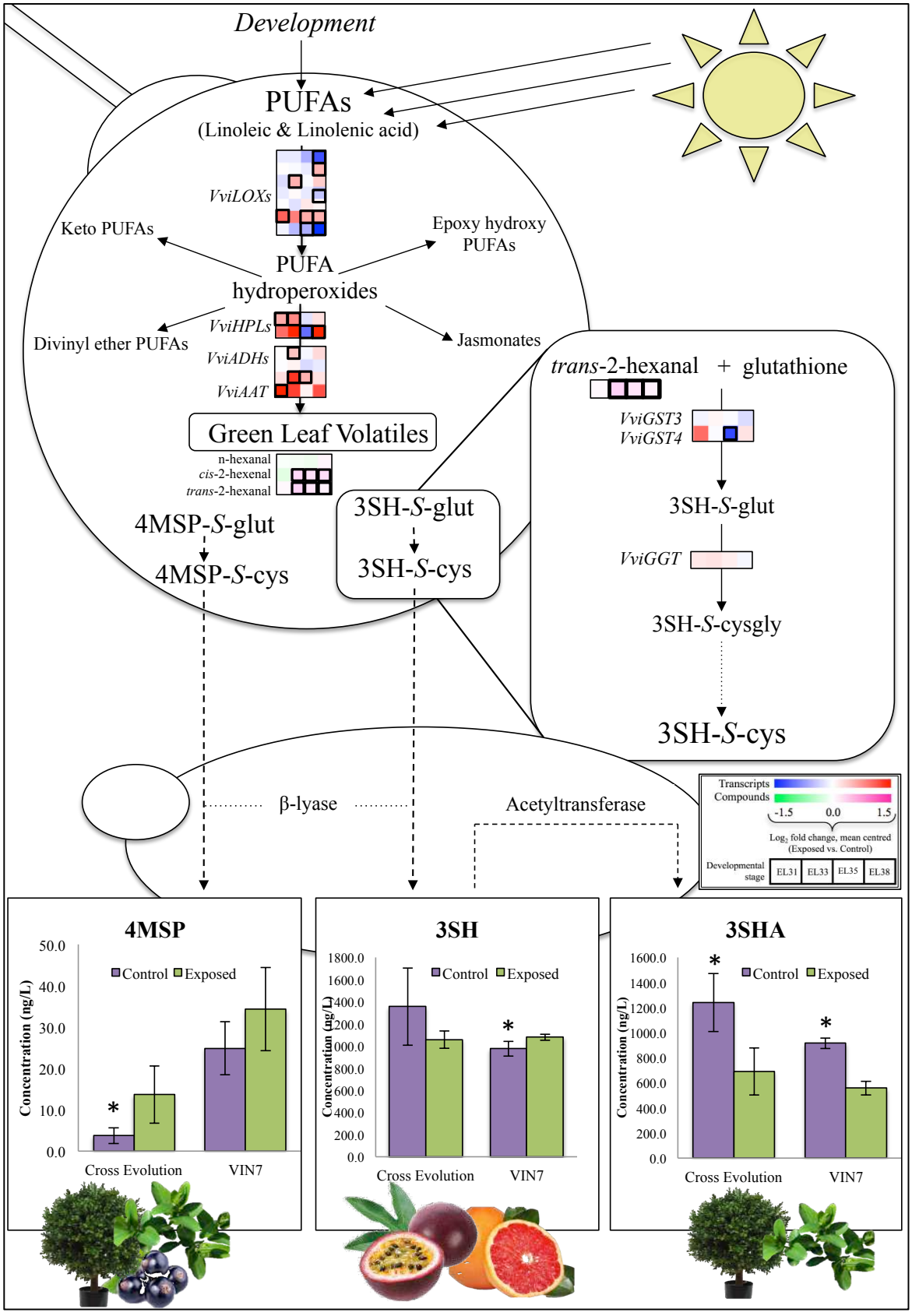
Development
PUFAs
(Linoleic & Linolenic acid)
VviLOXs
Keto PUFAs
Epoxy hydroxy PUFAs
PUFA hydroperoxides
VviHPLs
VviADHs
VviAAT
Divinyl ether PUFAs
Jasmonates
Green Leaf Volatiles
n-hexanal
cis-2-hexenal
trans-2-hexanal
4MSP-S-glut
4MSP-S-cys
3SH-S-glut
3SH-S-cys
trans-2-hexanal + glutathione
VviGST3
VviGST4
3SH-S-glut
VviGGT
3SH-S-cysgly
3SH-S-cys
β-lyase
Acetyltransferase
Transcripts
Compounds
-1.5
0.0
1.5
Log2 fold change, mean centred (Exposed vs. Control)
Developmental stage
EL31
EL33
EL35
EL38
4MSP
3SH
3SHA
Concentration (ng/L)
Control
Exposed
Cross Evolution
VIN7

Figure 6.3. The putative mechanisms involved in the development of volatile thiols from the degradation of polyunsaturated fatty acids (PUFAs) in grapes. A: The metabolic pathway and the genes putatively involved in the degradation of PUFAs through the expression of lipoxygenase encoding genes towards the synthesis of Green Leaf Volatile (GLVs) compounds that ultimately serve as precursors for the synthesis of non-volatile *S*-glutathionylated and *S*-cysteinylated thiol precursors in developing Sauvignon Blanc grape berries exposed to elevated light. Blocks indicate the mean-centered $\log_2$ fold change value when comparing the expression values (FPKM) of the transcripts putatively involved at each metabolic step. Significant differences ($q \leq 0.05$) are indicated by a bold contour (frame). B: A simplified representation of the metabolic process during which the grape *S*-cysteinylated thiol precursors are volatilized through the activity of yeast β-lyase during alcohol fermentation and the conversion of 3SH to 3SHA through yeast acetyltransferase activity. C: The concentrations (ng/L) of three volatile thiols measured in Sauvignon Blanc wines made from grapes grown under control conditions and elevated light exposure using two separate commercial *S. cerevisiae* yeasts (VIN7 and Cross evolution). Significant differences ($p < 0.05$) are indicated by an *.

6.4 Discussion

6.4.1 Elevated light significantly impacts the synthesis of Methoxypyrazines during early grape development

Methoxypyrazines collectively describe a small group aromatic compounds that are known to contribute to the herbaceous and vegetative aroma characteristics of wines made from specific *V. vinifera* cultivars (Robinson et al., 2014). Although these methoxypyrazines are frequently studied in the context of wine quality, their function in the context of grape physiology and development is associated with anti-herbivory and protection of the grapes against predators during the early grape developmental stages when the grape seeds are immature and developing. Thereafter, when the berries begins to ripen, the concentrations of these methoxypyrazines decrease rapidly, hereby contributing to making the grapes more palatable and attractive to dispersers of the mature grape seeds (Dunlevy et al., 2013; Hashizume et al., 2001). Furthermore, it is well understood that climate, light and temperature exposure of the developing grapes play a critical role in the final methoxypyrazine concentration of the grapes (Belancic and Agosin, 2007) although the mechanisms of their degradation remains poorly understood.

In this investigation, we explored the expression of the four known genes responsible for the final metabolic step in the synthesis of the methoxypyrazine, IBMP, in developing Sauvginon Blanc grapes. Our results revealed that all of these genes were expressed in green berry pulp and that *VviOMT1* and *VviOMT3* showed high levels of expression during the same stage (Figure A6.1; Addendum A to Chapter 6). Furthermore, we showed that *VviOMT1* and *VviOMT2* expression was highly conserved across eleven cultivars being investigated. The expression of *VviOMT3* and *VviOMT4* was, however, not detected in any of the cultivars other than Sauvginon Blanc (Figure A6.2; Addendum A to Chapter 6) and in comparing the sequences of these genes in the Pinot noir genome and the Sauvignon Blanc transcriptome, these genes were shown to be putative chimeras in Sauvignon

Blanc (Figure A6.3; Addendum A to Chapter 6). Interestingly, the activity of *VviOMT3* against both IBHP and 3-isopropyl-2-hydroxypyrazine (IPHP) was reported to be up to ~5000 fold higher than that of either *VviOMT1*, *VviOMT2* or *VviOMT4* in Cabernet Sauvignon (Dunlevy et al., 2010, 2013; Guillaumie et al., 2013).

Our data further provided evidence that elevated light exposure significantly downregulated the expression of all of these *VviOMTs* during the early stages of berry development. Furthermore, it has been well-established that elevated light exposure accelerates and elevates the degradation of these methoxypyrazine compounds since they are sensitive to photodecomposition, hereby reducing herbaceous notes in wines made from Sauvginon Blanc grapes (reviewed in Sidhu et al., 2015). Combining these results showing lower *VviOMT* expression and our knowledge regarding methoxypyrazine decomposition in response to elevated light, it would be reasonable to hypothesize that wines made from these exposed grapes would have lower methoxypyrazine compositions and lower vegetal aroma content than control grapes. Although the methoxypyrazine concentrations of the wines were not evaluated in this study, the transcriptional data is supported by findings reported from subsequent harvests from the same vines under exposed and control conditions (Šuklje et al., 2016). The authors presented data indicating that the leaf removal treatment significantly reduced the concentration of the methoxypyrazine, IBMP, in the wines made from the exposed grapes and that the wines were perceived as less "green", with elevated fruity aromas.

6.4.2 Elevated light affects the accumulation of thiol precursors by upregulating genes involved in GLV synthesis.

Similar to methoxypyrazines, GLVs play an indispensible role in plant defense strategies by either repelling phytopathogens directly (Nakamura and Hatanaka, 2002; Prost et al., 2005), or by attracting predators of the threatening pathogens in order to defend itself against the pathogen indirectly (Halitschke et al., 2008; Kessler and Baldwin, 2001; Schuman et al., 2012; Shiojiri et al., 2006). Physiologically, the glutathionylated thiol precursors are involved in the detoxification of oxidative byproducts formed in response to plant responses to biotic and abiotic stresses (Peyrot des Ganchons et al., 2002).

In the synthesis of these GLVs, the main substrates for lipoxygenase enzymes are C_{18} PUFAs predominantly represented by linoleic and α-linolenic acid (Blée, 1998). These LOX enzymes are responsible for the first enzymatic step in the oxygenation of PUFAs and thereby result in the production of either 13(S) or 9(S)- PUFA hydroperoxides depending on which LOX enzyme catalyzed the reaction. The results generated in this investigation revealed that among the ten *VviLOXs* previously characterized (Podolyan et al., 2010), only one of these genes, *VviLOXA*, was constitutively expressed in all of the grape berry tissues explored through RNASeq analyses (Figure A6.1; Addendum A to Chapter 6). *VviLOXA* encode for a 13-LOX and is therefore responsible for the synthesis of 13(S)-hydroperoxides that are known substrates for the downstream enzymatic activity of

HPL enzymes. In this investigation, one of the HPL encoding genes, *VviHPLA*, also showed constitutive expression in all berry tissues throughout development (Figure A6.1; Addendum A to Chapter 6).

These findings are consistent with an earlier study in which *VviLOXA* was reported to be the most abundantly expressed *LOX* gene in the grapevine genome (Podolyan, 2010). This earlier study did however report that *VviLOXA* was predominantly expressed in berry skins, contrasting to our findings that revealed its expression in all berry tissues throughout development (Figure A6.1; Addendum A to Chapter 6). This discrepancy in results being reported for *VviLOXA* expression might be attributed to the fact that this gene shows very low sequence similarity when comparing the Pinot noir reference genome and the *de novo* assembled transcriptome for Sauvignon Blanc (Table A6.1; Addendum A to Chapter 6). The expression of this gene in Sauvignon Blanc reported in the earlier study (Podolyan, 2010) was based on semi-quantitative real-time PCR results generated with primers designed based on the reference genome (PN40024) and the extent of the sequence differences between the two cultivars may account for this discrepancy. In support of our findings, both *VviLOXA* and *VviHPLA* were also expressed in all ten Italian cultivars investigated through RNASeq, although the expression of *VviLOXA* was consistently expressed at higher levels in developing Sauvignon Blanc grapes (Figure A6.2; Addendum A to Chapter 6). In addition, *VviLOXA*, *VviHPLA*, *VviADHs* and *VviAAT* were significantly upregulated in response to higher levels of light during the early berry developmental stages, potentially resulting in the accumulation of higher levels of two of the GLVs observed (Figure 6.3).

The genes involved in the down-stream synthesis of cysteinylated precursors to the 3SH volatile thiol, including *VviGST3* and *VviGGT*, were found to be not only consistently expressed in Sauvignon Blanc grapes, but in each of the ten Italian cultivars as well (Figure A6.2; Addendum A to Chapter 6). The expression of these genes were, however, not affected by elevated light exposure in the Sauvginon Blanc samples, but because there were significantly higher levels of the GLVs, *trans*-2-hexanal and *cis*-2-hexenal, followed by significant differences in the concentrations of the volatile thiols 3SH and 3SHA between wine made from control and light exposed grapes, it would be reasonable to expect that there were in fact more cysteinylated 3SH precursors in the ripe berries exposed to elevated light (for review, see Coetzee et al., 2012). Based on these findings, the final metabolic steps involved in the synthesis of non-volatile 3SH precursors are potentially not transcriptionally regulated by elevated light, but rather by elevated levels of the GLV substrates as a result of the upregulation of *VviLOXA* and *VviHPLA* in response to the treatment (Figure 6.3).

Interestingly, the expression of *VviGST4* was consistent in ripening berries of all of the red Italian cultivars but not in any of the white cultivars (Figure A6.2; Addendum A of Chapter 6) and this transcript could not be identified in the newly assembled Sauvginon Blanc transcriptome (Table A6.1; Addendum A of Chapter 6). These findings may have interesting consequences in future studies focused towards the synthesis of volatile thiols in red cultivars.

In this investigation, wine was produced from light exposed and control grapes using two separate yeast strains that had distinctly different results. Although the concentrations of 4MSP were consistently higher and 3SHA was consistently lower in wine made from light exposed grapes, the accumulation of 3SH differed depending on which yeast strain was used (Figure 6.3).

It is well known that the *S. cerevisiae* yeast strain with which grape juice is inoculated play a significant role in the ultimate thiol composition of the wines (Belda et al., 2016; Dubourdieu et al., 2006; Houtman and Du Plessis, 1986; Howell et al., 2004, 2005; Renault et al., 2015) and that juices with more thiol precursors do not necessarily lead to the accumulation of higher levels of thiols in wines (Capone et al., 2010; Roland et al., 2011). In a study focused on the fermentation of Sauvignon Blanc juices, the thiol concentrations of wines produced with seven commercial *S. cerevisiae* strains were compared (Swiegers et al., 2009). The authors reported the significant differential ability of specific wine yeast strains to liberate 3SH from its precursor conjugates and that one of the yeast strains used in our study, VIN7, produced the highest concentrations of volatile thiols, similar to the findings reported here. Subsequent studies have revealed that specific yeast strains, including non-*Saccharomyces* strains, have the capacity to only convert glutathionylated precursors, whereas others are limited to the conversion of cysteinylated precursors (Renault et al., 2016), hereby pointing to the benefit of yeast co-inoculations for the purpose of optimizing thiol accumulation in wines. Furthermore, the underlying genetic characteristics associated with this differential ability to convert thiols are further being investigated (for review see Coetzee et al., 2012) and will significantly enhance our current ability to anticipate thiol concentrations from the onset of grape development to the final product in the wine.

6.4.3 Conclusions and future prospects

The contributions of both methoxypyrazines and volatiles thiol precursors synthesized in developing Sauvignon Blanc grapes have been extensively explored. However, to our knowledge, the underlying transcriptional patterns associated with both methoxypyrazine and thiol precursor synthesis in Sauvignon Blanc grapes have not been evaluated simultaneously and the effect of elevated light on the associated gene expression remained to be determined.

In this study, we determined that although the expression of the genes involved in both thiol precursor and methoxypyrazine synthesis appears to be highly conserved among all the grape cultivars investigated here, the *de novo* assembly of the Sauvignon Blanc transcriptome revealed that several of these genes show low sequence similarity to the Pinot noir reference genome. Some of these transcripts identified in the Sauvignon Blanc transcriptome mapped to more than one chromosome or to more than one location on the same chromosome in the *V. vinifera* genome assembly (12X) and are therefore considered to be putative chimeras. These sequence

differences may have a profound effect on the varietal aroma composition when comparing Sauvignon Blanc wines to those of other cultivars and warrant further investigation.

As part of the synthesis of thiol precursors, elevated light exposure was shown to effect only a small subset of the genes involved in the synthesis of GLVs and that the genes associated with the down-stream synthesis of cysteinylated thiol precursors were not affected by elevated light. Our findings further supported the fact that the chosen wine yeast strain significantly affects the final accumulation of volatile thiols in Sauvignon Blanc wines. The accumulation of methoxypyrazines was predicted to accumulate at much lower concentrations in grapes exposed to elevated light through two metabolic steps. Firstly, the genes responsible for the synthesis of methoxypyrazines was significantly downregulated by light exposure and secondly, from previous reports, it is well established that elevated light increases the degradation of methoxypyrazines during berry ripening.

Therefore, based on the transcriptomic evidence supported by the thiol concentrations of the resulting wines, elevated light in the bunch zone of developing Sauvignon Blanc grapes will produce wines with lower vegetal/herbaceous characteristics due to combined lower levels of both methoxypyrazines and the 3SHA volatile thiols due to the underlying transcriptional effect of the elevated light treatment. The strongly elevated levels of norisoprenoids and terpenes that accumulated in these grape berries at harvest (Young et al., 2016; Addendum B to Chapter 3) would further contribute to lower perceived greenness of the wines as a result of the photoprotective mechanisms implemented by the grapes to mitigate the effects of elevated light exposure. Future studies focused towards the accumulation of volatile thiols, their non-volatile precursors and methoxypyrazine concentrations in each of the grape-derived matrices (developing grapes, juice, must and wine) will further link the expression of the associated genes and ultimate accumulation in Sauvignon Blanc varietal aroma compounds grapes and wine.

6.5 References

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Addendum A to Chapter 6

This Addendum contains relevant and additional data not shown in Chapter 6.

A6.1 Materials and Methods

A6.1.1 The expression patterns of genes known to be involved in the metabolism of aroma compounds in grape berry tissues

The expression patterns of the genes being investigated in this study were explored in various tissues and cultivars. These analyses were preformed by firstly comparing RNASeq data generated for the developing Sauvignon blanc grapes (whole berry pericarp, skin and pulp separately) under control conditions. Secondly, in order to explore the cultivar-specific expression patterns of these genes, RNASeq data generated in this investigation was compared to the expression of the same genes in 5 red and 5 white Italian cultivars as generated by RNASeq analysis (Massonnet et al., 2017). The expression values of all of the genes putatively involved in the synthesis Sauvignon blanc aroma compounds were normalized separately by dividing each expression value with the average expression value calculated for each of the sixteen genes within each experiment, respectively. Hereby, the ratio of expression within each experiment could be effectively compared between different experiments by taking the inherent differences between the experimental methods and/or practices into account. The expression in various grapevine tissues and cultivars were represented in the form of heat-maps generated in the Multi-Experiment Viewer (MeV; Saeed et al., 2006).

A6.2 Results

A6.2.1 The expression patterns of the genes involved in the synthesis of methoxypyrazines and thiol precursors

A6.2.1.1 Tissue specific expression patterns in developing berries

In order to determine the tissue-specific expression patterns of each of the sixteen target genes, these genes were identified in the RNASeq data results generated for whole berries (pericarp), skin and pulp sampled during the green, veraison and ripening stages of the development of these Sauvignon blanc grapes under normal growing conditions (Figure A6.1). These results reveal that only one of the characterized LOX encoding genes, *VviLOXA*, showed consistent constitutive expression in all grape berry tissues throughout the developmental program of the grapes. Two other LOX encoding genes, *VviLOXO* and *VviLOXL*, showed developmental stage-specific expression whereas most of the other *VviLOXs* were not expressed in any of the grape berry tissues evaluated.

Out of the two *VviHPL* genes targeted in this study, only *VviHPLA* showed high levels of expression and *VviHPLF* was not expressed in any grape berry tissues. Similarly, in the downstream metabolism of GLVs towards the synthesis of thiol precursors, one Glutathione S-transferase (*VviGST3*) showed consistently high levels of expression whereas the other, *VviGST4*, was not expressed at all. The gene responsible for the final metabolic step towards 3MH precursor synthesis, *VviGGT* (Gamma-glutamyltranspeptidase), showed negligibly low expression levels in all green berry tissues but expression consistently increased by véraison and reach high levels of expression in all ripening berry tissues.

The O-methyltransferase encoding genes responsible for the synthesis of methoxypyrazines consistently showed no expression after the green developmental stages in any of the berry tissues. Two of these genes, *VviOMT1* and *VviOMT3*, showed high levels of expression in whole berry and pulp tissue samples and to a lesser degree in skin samples, whereas the other two *VviOMTs* were similarly expressed in whole berries and pulp, but not in skin samples (Figure A6.1).

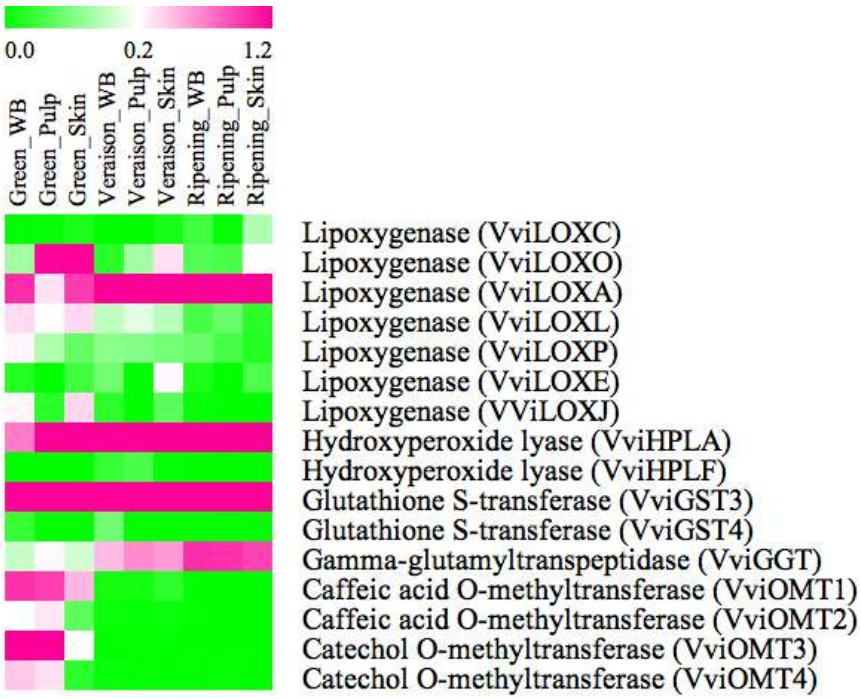

Figure A6.1. A heat map depicting the normalized* tissue-specific expression of the genes putatively involved in Sauvignon blanc varietal aroma synthesis in whole berries (pericarp), skin and pulp throughout development. * refers to the expression ratio relative to each separate experiment (described in materials and methods).

A6.2.1.2 Cultivar specific expression patterns

To determine whether the expression of the sixteen target genes putatively involved in the synthesis of Sauvignon blanc varietal aroma compounds show unique patterns in other cultivars, the RNASeq data generated from this investigation from whole Sauvignon blanc grapes were compared to RNASeq data generated from developing grapes of ten Italian cultivars (Massonnet et al., 2017). In the synthesis of thiol

precursors, the expression of these genes were predominately similar between all of the cultivars investigated, however, some interesting differences did emerge (Figure A6.2). Among the LOX encoding genes, *VviLOXO* was expressed at substantially higher levels in all of the ten Italian cultivars compared to Sauvignon blanc during the green berry developmental stages, whereas *VviLOXA* appeared to expressed at consistently higher levels during all developmental stages is Sauvignon blanc. Although two of the other LOXs, *VviLOXL* and *VviLOXP*, was expressed at slightly higher levels in the ten Italian cultivars, the expression patterns were mostly similar when comparing all the cultivars to Sauvginon blanc. When comparing genes involved in the down-stream synthesis of thiol precursors, the expression of *VviGST3* and *VviGGT* appeared to be highly conserved among all the cultivars, however, *VviGST4* showed clear differences with high levels of expression reported in all the red cultivars compared to absolutely no expression in any of the six white cultivar investigated.

In the synthesis of methoxypyrazines, the expression of *VviOMT1* and *VviOMT2* was highly similar among all of the cultivars and showed expression in all of the ten Italian cultivars during both of the green developmental stages even though the expression of these genes appeared to have decreased more dramatically after EL31 in Sauvignon blanc grapes. Furthermore, the expression of *VviOMT3* and *VviOMT4* were not reported in any of the ten Italian cultivars despite showing expression in green Sauvignon blanc grapes (Figure A6.2).

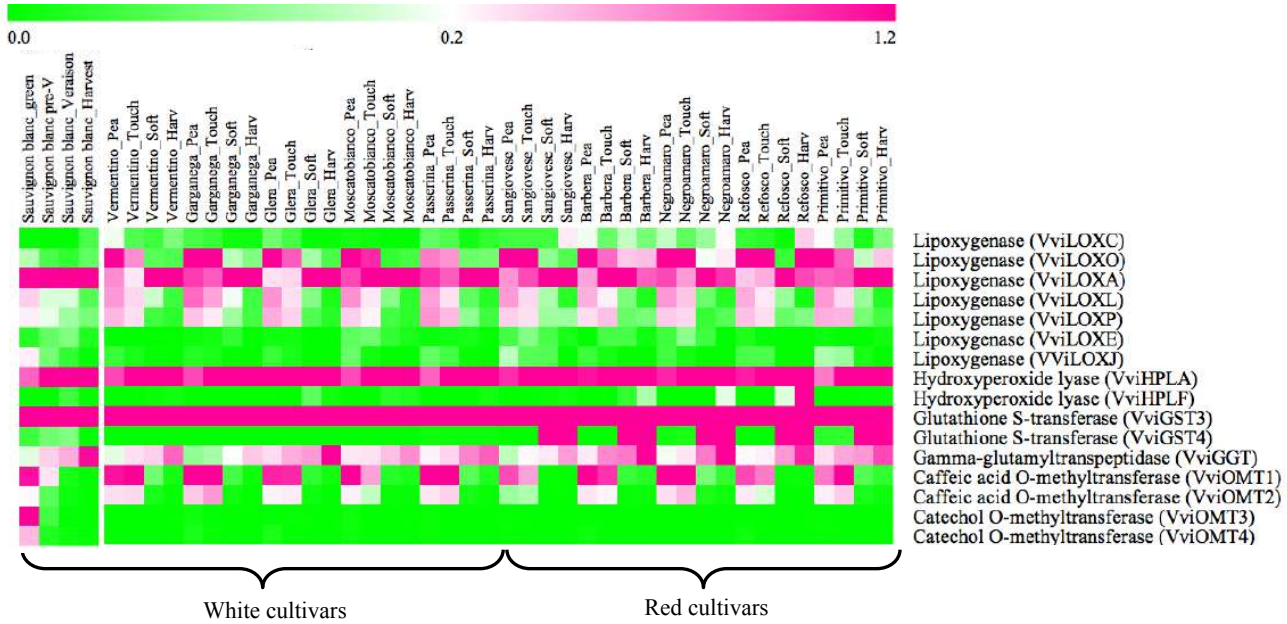

Figure A6.2. Heat maps comparing the relative* expression of the genes putatively involved in Sauvignon blanc varietal aroma synthesis in developing berries from Sauvignon blanc, five white and five red Italian cultivars (Massonnet, 2015).
* refers to the expression ratio relative to each separate experiment (described in materials and methods).

Due to the fact that most RNASeq sequence reads are aligned to the Pinot noir reference genome, we attempted to determine the level of sequence identity when comparing these target genes in the *de novo* assembled Sauvginon blanc transcriptome. Based on the results from nucleotide BLAST analyses, it was

determined that most of the genes putatively involved in the synthesis of Sauvignon blanc aroma characteristics showed relatively low sequence similarity between then Pinot noir and Sauvignon blanc sequences (Table A6.1). Among the ten *VviLOX* genes, only three could be successfully identified with complete coding sequences (CDS) and showed a high level of sequence identity between the two cultivars (>99%). Three of these LOXs, including *VviLOXC*, *VviLOXH* and *VviLOXG*, could not be identified in the Sauvginon Blanc transcriptome, with another four of these LOX genes either aligning to multiple hits or showing poor CDS alignment in the Sauvignon Blanc transcriptome. The two down-stream HPL encoding genes in the Sauvginon blanc transcriptome aligned uniquely to their corresponding sequences with high sequence identity in the reference genome despite achieving only partial CDS alignments. Furthermore, *VviGST4* could not be identified in the Sauvginon blanc transcriptome even though the other two genes, *VviGST3* and *VviGGT*, aligned completely and showed very high levels of identity (>99%) when comparing their sequences in the two cultivars.

None of the genes encoding the *VviOMTs* involved in methoxypyrazine synthesis could be uniquely aligned in the Sauvginon Blanc transcriptome despite showing complete CDS alignment (Table A6.1; Figure A6.3). According to these findings, *VviOMT1* and *VviOMT2* are in fact putative chimeras in the Sauvginon blanc transcriptome. This was also found to be true for *VviOMT3* and *VviOMT4* further showing relatively low sequence similarity to the nucleotide sequences in the Pinot noir genome (Table A6.1; Figure A6.3).

Table A6.1. Details pertaining to the genes putatively involved in the synthesis of Sauvignon blanc varietal aroma compounds represented in the *de novo* transcriptome assembly of Sauvignon blanc in comparison to the Pinot noir reference genome (PN40024). The genes that were either not present in the Sauvignon blanc transcriptome, had multiple hits or could only be partially confirmed based on their coding sequences (CDS) are shaded in grey.

Pathway involved	Gene name	Sauvignon blanc assembly accession	Nucleotide length	Protein length	Mapping on the *V. vinifera* genome	Sauvignon blanc identity (%)
Lipoxygenase pathway	*VviLOXC*	Not found	-	-	-	-
	VviLOXO	EVGmRNA001594t1	3385	916	uniquely mapping	99
	VviLOXA	EVGmRNA015371t1	2281	286	putative chimeras	87
	VviLOXH	Not found	-	-	-	-
	VviLOXL	EVGmRNA001836t1	3744	876	uniquely mapping	99
	VviLOXP	EVGmRNA001571t1	3304	920	uniquely mapping	99
	VviLOXI	EVGmRNA003670t1	3553	682	multiple hits	85
	VviLOXE	EVGmRNA018371t1	2541	226	multiple hits	94
	VviLOXJ	EVGmRNA003670t1	3553	682	multiple hits	81
	VviLOXG	Not found	-	-	-	-
	VviHPLA	EVGmRNA007574t1	2474	487	uniquely mapping	98
	VviHPLF	EVGmRNA006495t1	1571	523	uniquely mapping	99
2MH-S-cys synthesis pathway	*VviGST3*	EVGmRNA019015t1	1070	216	uniquely mapping	99
	VviGST4	Not found	-	-	-	-
	VviGGT	EVGmRNA004433t1	2195	626	uniquely mapping	99
Methoxypyrazine pathway	*VviOMT1*	EVGmRNA011411t1	1387	373	putative chimeras	99
	VviOMT2	EVGmRNA011411t1	1387	373	putative chimeras	96
	VviOMT3	EVGmRNA012027t1	1778	359	putative chimeras	97
	VviOMT4	EVGmRNA012027t1	1778	359	putative chimeras	88

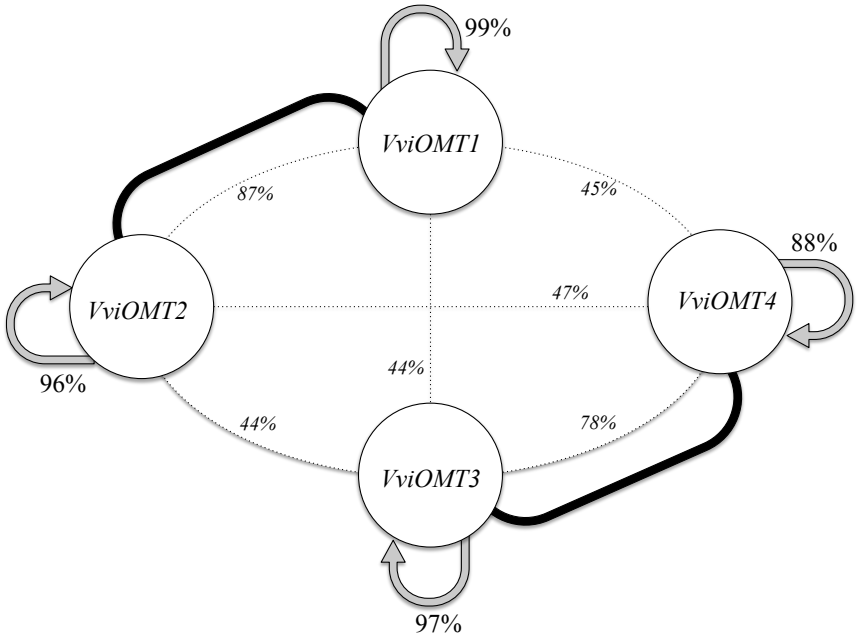

Figure A6.3. The nucleotide sequence relationship between *VviOMTs* in the Pinot noir reference genome and the Sauvginon blanc transcriptome. Dotted lines represent the percentage (%) sequence similarity between the respective *VviOMTs* in the reference genome. Grey arrows indicate the percentage identity (%) when comparing the Sauvignon blanc transcriptome to the Pinot noir reference genome. Black bars indicate the putative chimeras reported in the Sauvginon Blanc transcriptome.

A6.3 References

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Addendum B to Chapter 6

This Addendum contains relevant and additional data not shown in Chapter 6

B6.1 The effect of elevated light exposure on methoxypyrazine synthesis in developing Sauvignon Blanc grapes.

Table B6.1. The effect of elevated light exposure on the expression and accumulation of genes and compounds in the metabolic pathway involved in the synthesis of methoxypyrazines. Values indicate the log_2 fold change when comparing the expression levels (FPKM values) or the concentrations (ng/L) measured between exposed and shaded grapes at each developmental stage. Significant differences are indicated in bold and are italicized.

		Log_2 Fold change (Exposed vs Control)			
Gene/Compound	Gene annotation (12X)	EL31	EL33	EL35	EL38
VviOMT1	*VIT_12s0059g01790*	***-0.59***	***-1.18***	-0.42	0.23
VviOMT2	*VIT_12s0059g01750*	***-0.76***	***-1.15***	-0.76	-0.45
VviOMT3	*VIT_03s0038g03090*	-0.24	-0.28	-0.05	0.32
VviOMT4	*VIT_03s0038g03080*	***-0.49***	-0.03	0.59	-0.57
Leu		-0.77	0.16	-0.23	0.10
Gly		***1.55***	***1.15***	***0.98***	***0.31***

B6.2 The effect of elevated light on the synthesis of thiol precursors in developing grapes and its possible link to thiol concentrations in Sauvignon blanc wine

Table B6.2. The effect of elevated light exposure on the expression and accumulation of genes and compounds in the metabolic pathway involved in PUFA degradation and the accumulation of thiol precursors. Values indicate the log_2 fold change when comparing the expression levels (FPKM values) or the concentrations (ng/L) measured between exposed and shaded grapes at each developmental stage. Significant differences are indicated in bold and are italicized.

			Log2 Fold change (Exposed vs Control)			
Pathway involved	Gene/Compound	Gene annotation (X12)	EL31	EL33	EL35	EL38
Lipoxygenase pathway	Lipoxygenase *(VviLOXC)*	*VIT_14s0128g00780*	-0.17	-0.17	-0.60	***-1.23***
	Lipoxygenase *(VviLOXO)*	*VIT_09s0002g01080*	0.02	-0.05	-0.17	***0.54***
	Lipoxygenase *(VviLOXA)*	*VIT_06s0004g01510*	-0.26	***0.56***	-0.05	0.37
	Lipoxygenase *(VviLOXL)*	*VIT_05s0020g03170*	-0.16	0.04	-0.18	***-0.45***
	Lipoxygenase *(VviLOXP)*	*VIT_01s0010g02750*	-0.02	-0.23	0.17	0.06
	Lipoxygenase *(VviLOXE)*	*VIT_06s0004g01450*	***1.07***	0.89	***0.59***	***0.56***
	Lipoxygenase *(VViLOXJ)*	*VIT_13s0064g01480*	-0.10	-0.52	***-0.59***	***-1.75***
	Hydroxyperoxide lyase *(VviHPLA)*	*VIT_12s0059g01060*	***0.61***	***0.83***	-0.22	0.23
	Hydroxyperoxide lyase *(VviHPLF)*	*VIT_03s0063g01820*	0.00	5.33	***-1.04***	***3.47***
	Alcohol dehydrogenase *(VviADH1)*	*VIT_18s0001g15410*	0.01	***0.44***	-0.06	0.28
	Alcohol dehydrogenase *(VviADH2)*	*VIT_18s0001g15450*	-0.02	0.10	-0.29	-0.16
	Alcohol dehydrogenase *(VviADH3)*	*VIT_04s0044g01120*	0.24	***1.34***	***0.56***	0.22
	Alcohol acyl transferase *(VviAAT)*	*VIT_00s0187g00260*	***2.32***	1.27	0.10	1.18
3MH-*S*-cys synthesis pathway	Glutathione S-transferase *(VviGST3)*	*VIT_12s0028g00930*	-0.09	0.06	-0.03	-0.27
	Glutathione S-transferase *(VviGST4)*	*VIT_04s0079g00690*	0.00	-0.04	***-1.28***	0.21
	Gamma-glutamyltranspeptidase *(VviGGT)*	*VIT_11s0016g02830*	0.17	0.21	0.13	-0.07
	Trans-2-hexanal		0.06	***0.34***	***0.20***	***0.11***
	N-Hexanal		-0.05	-0.09	-0.13	0.08
	Cis-2-Hexenal		-0.22	***0.30***	***0.23***	***0.26***

Chapter 7

General discussion and conclusions

Chapter 7

General discussion and conclusions

7.1 General discussion

The integration of several omics disciplines in the study of the complex metabolic responses of grape berries to specific abiotic stresses have gained interest in recent years. However, due to the complexities associated with separating the effects of temperature and light in a field setting, the effect of light in the grape berry microclimate has been rarely studied in a multi-omics context.

Before the commencement of this PhD project, the first study that was conducted in the same model vineyard targeted specific metabolites for analysis. This earlier study initially determined that the grapes were not physically different when comparing shaded and light exposed grapes but that specific secondary metabolites were altered in response to the treatment. Subsequently, in support of these initial findings, RNASeq data generated as part of this PhD study was implemented in targeting the genes involved in the pathways that were metabolically altered (Young et al., 2016; Addendum B of Chapter 3). From the results generated in this preceding study, a new question arose:

How were primary metabolism and developmental patterns maintained, despite the light stress-response and metabolic reorganization activated in grape berries exposed to elevated light?

This question prompted the formulation of several smaller questions in order to systematically address the gaps in our knowledge regarding how grape berries respond to elevated light exposure. These research questions are stipulated below and addressed in the sections to follow.

1. Was our approach successful at establishing confidence in the causality of the leaf removal treatment on the transcriptional and metabolic results generated from this study? (Addressed in section 7.1.1)
2. Could it be confirmed that the grapes exposed to elevated light was effective at acclimating to the elevated light exposure? (Addressed in section 7.1.2)
3. Through which metabolic mechanisms did these grapes achieve acclimation to elevated light? (Addressed in section 7.1.3)
4. What regulatory mechanisms were involved in the achievement of this acclimated state? (Addressed in section 7.1.4)

7.1.1 The field-omics approach is successful at more accurately linking transcriptional responses to specific microclimatic conditions

In the process of studying the complexity of plant metabolism on a molecular level, the success of a specific experimental approach can be determined by accurately linking the impact of a treatment on the results and the repeatability thereof. The term "field-omics" was put forward in Alexandersson et al., (2014) to describe an experimental approach that could potentially be effective at mitigating the effects that highly variable field conditions may have on the outcome of crop studies. This approach involves the comprehensive characterization of the environmental and growing conditions of the crop being studied to understand and recognize potential sources of unintended variability, before the integration of data layers started. This is particularly important when implementing field studies with omics techniques that provide snapshots in time of transcripts, proteins and metabolites that are strongly responsive to the environmental influences. Acknowledging these complexities and adopting appropriate experimental workflows is paramount to effectively determine the causality of the effect of the treatments/conditions being studied (Alexandersson et al., 2014). By acknowledging the importance of meticulous experimental planning and multi-omics integration, recent studies have had great success with this approach and have contributed to the improvement of grapevine field experimental systems (Carbonell-Bejerano et al., 2016; Reshef et al., 2017). Furthermore, the effectivity of these highly integrated approaches was evident in the context of how grapes respond to salinity stress (Daldoul et al., 2014).

The work presented in this thesis convincingly showed that by implementing a field-omics approach, high levels of repeatability between biological replicates and the effective establishment of the impact of a treatment can be achieved. The replicates that were utilized in the RNASeq analysis exceeded general expectations associated with the repeatability of field-grown biological replicates. This observation is evident in Chapter 3 (Figure 1) in which the correlation of the full transcriptomes of each of the replicates were visualized, clearly confirming the high level of repeatability between samples taken at each developmental stage. On the metabolic level, the results generated over several vintages in the same vineyard were consistent (Addendum B of Chapter 3), hereby further eliminating the "vintage effect" to allow for more conclusive links to be drawn between the implemented treatment and the observed implications.

The confirmation of causality of a specific treatment remains one of the greatest challenges in the study of grapevines grown in the highly variable vineyard environment. By extensive characterization of the macro-, meso- and microclimatic conditions of the grapes, it could be concluded that the levels of light exposure that these grapes experienced were the main measured variable that was significantly affected by the leaf removal treatment. It would be unreasonable to expect that no other variables could have impacted on the development of these grapes, however, most of the well-known impact factors, such as wind exposure, temperature, water deficit and disease pressures were quantified as part of the characterization of this model vineyard. This diligent characterization instilled confidence in the downstream analysis performed with

these grapes (Young et al., 2016; Addendum B of Chapter 3). Further testament to the effect of the light treatment, was the fact that several instances revealed differential gene expression and metabolic consequences concordantly in response to the light treatment. Among the many examples of this concordance was revealed in Chapter 3 (Figure 8) where it was shown that the upregulation of the flavonol-synthase encoding gene (*VviFLS1*) coincided with developmentally independent accumulation of flavonols in response to elevated light.

The repeatability and causality of the implemented treatment serve as a reflection of thorough experimental planning and implementation at the commencement of the greater research project of which this PhD formed part. This success is rooted in the fact that each layer of information generated from the highly characterized vineyard provided valuable insights for planning subsequent analyses. For example, after measurements of the climatic factors, physical parameters and targeted metabolites were conducted over a three-year period, the biological replicates that behaved with the lowest levels of statistical variability could be identified for the purpose of RNASeq analysis (Addendum A to Chapter 3). Subsequently, the RNASeq results revealed which primary and secondary metabolic processes appeared to be altered on a transcriptional level, hereby guiding the selection of downstream metabolite analyses toward the measurement of amino acid and phenolic compound concentrations. Consequently, these findings could therefore provide valuable information to future research focused on the development of specific varietal aroma compounds in wines made from the same grape samples.

7.1.2 The grape berries successfully acclimated to elevated light as evident by the fact that development remained the strongest driver in grape berry gene expression regardless of treatment.

The initial characterization of the grapes revealed that light exposed and shaded (control) grapes were not physically different based on their weight, diameter and ripening parameters such as the accumulation of sugars and organic acids (Young et al., 2016; Addendum B of Chapter 3). In this thesis, whole transcriptome analyses revealed that more than 90% of all the genes annotated in the grapevine genome remained unaffected by elevated light at every developmental stage evaluated (Figure 2; Chapter 3). These unaffected genes were predominantly involved in primary metabolic processes, hereby confirming that not only were the berries largely unaffected on a metabolite level (Young et al., 2016), but that the underlying molecular mechanisms associated with primary metabolism remained constant as well (Chapter 3).

Chapter 4 further contributed to the establishment of grape berry developmental stage-specific biomarkers that were similar in previous investigations (Zamboni et al., 2010; Palumbo et al., 2014) and were not affected by the light treatment. The identification of these biomarkers not only confirmed the normal progression of the grape development in this study, but contributed to the establishment of developmental biomarkers that could be utilized in future studies to establish the normal development of grapes under variable environmental conditions.

Traditionally, modulations in light exposure to developing grapes have been associated with higher levels of sugar accumulation and decreased acidity in the case of higher exposure (for review, see Reynolds et al., 2010) while lower berry weight and total soluble solids have been reported in ripe grapes exposed to low levels of light (Dokoozlian and Kliewer, 1996; Zhang et al., 2014; 2017). However, none of these grape parameters were affected in this study and therefore our findings further support the initial hypothesis that the grapes developing under elevated light conditions were effectively acclimated to the abiotic stress condition. We therefore assumed that the light stimulus was not perceived as a stress. This is, however, a weighted assumption upon which several subsequent conclusions were elaborated. Reactive oxygen species (ROS), such as H_2O_2, are known as the most conclusive biotic and abiotic stress-related markers in plants. In this study, however, ROS was not measured and therefore, the stress-status of the berries at each developmental stage were implied but were not conclusively confirmed. Other indicative stress markers such as proline and GABA were used in this case to indicate whether the berries perceived the treatment as a stress, however, determining the levels of ROS accumulation would have instilled greater confidence in this hypothesis. Nonetheless, we concluded that the grape berries were effectively acclimated and that it was less than 10% of genes altered by the light treatment that achieved this acclimated state that was further investigated.

7.1.3 Green, photosynthesizing grape berries exposed to elevated light achieved a state of acclimation to maintain growth and development at all costs.

From this model vineyard project it was initially determined that the accumulation of xanthophylls were altered in response to elevated light exposure to the grapes and, not surprisingly, the genes involved in non-photochemical quenching (NPQ) were significantly upregulated as well (Young et al., 2016; Addendum B of Chapter 3). What was surprising, however, was that the genes encoding the proteins of the photosynthetic machinery were also significantly upregulated when the grapes were photosynthetically active, without exceptions (Figure 6; Chapter 3). These findings therefore established that highly responsive changes to the transcriptome were underlying very subtle changes to the metabolites involved. These thylakoid membrane proteins were therefore rapidly and continuously replaced with new copies to avoid damage to the photosynthetic machinery as part of reversible photoinhibition (RPI). Despite this highly active underlying photoprotective mechanism, elevated light exposure neither affected the accumulation of the primary light harvesting pigments (chlorophyll a and b as discussed in Young et al., 2016) nor the primary metabolism of the berries (as previously discussed in section 7.2.2). Taken together, these results point towards a metabolic "treadmill" effect, in which the proteins of the photosynthetic machinery are rapidly recycled in order to maintain a constant metabolic state.

This conclusion however, assumes that the net rate of photosynthesis was not affected in the exposed grapes and since this parameter was not quantified in this study, a relatively large leap had to be taken based on the

stability of the chlorophyll pigments to the conclusion that there was no effect on photosynthesis. Furthermore, the accurate quantification of photosynthesis rates in fruits is currently notoriously inefficient. However, if the rate of photosynthesis was affected by the dramatic upregulation of genes involved in photosynthesis, other mechanisms not explored in this study would have had to be involved in maintaining the ratios of chlorophyll pigments and ultimately the primary metabolism of the exposed grapes. Therefore, although this conclusion may have oversimplified the complexities involved in the photosynthetic machinery, it provided the initial holistic overview of how grapes respond to elevated light upon which future studies can elaborate.

The second layer at which the developing grapes were actively mitigating the effects of light stress, was in the transcriptional upregulation and metabolic accumulation of compounds with antioxidant and/or sunscreen capacity. Some of these compounds include the accumulation of flavonols, carotenoids (and apocarotenoids) and other lipophilic antioxidants that may include tocopherol. Despite the well-established role of these compounds in photoprotection, these mechanisms proved to be ineffective without the active collaboration of the photosynthetic "treadmill". This failure to successfully mitigate light stress was evident by the accumulation of stress-related biomarkers (proline and GABA) in ripe, non-photosynthetic grapes. In essence, the photosynthetic machinery proved to be crucial for effective acclimation to light stress since flavonols and apocarotenoids were not sufficient for stress mitigation after degradation of the photosynthetic machinery.

The cost accrued to this photosynthetic "treadmill" in parallel with the upregulation of energetically costly secondary metabolites (flavonols, carotenoids, apocarotenoids) becomes substantial when the berries were no longer photosynthesizing. It appears as though the green grapes invested all available resources (including amino acids) to stress mitigation, however, the effects of this resource depletion only became evident when photosynthetic activity/context declined. This observation hints towards the fact that these green berries are in fact not entirely sink organs, but retain the limited ability to contribute to the resources accumulated before photosynthesis ceases, similar to vegetative source organs, such as leaves, although fruit photosynthesis has very unique characteristics. This type of metabolic contribution to the acclimation of green fruit to environmental stress has been briefly reviewed in the distant past (Blanke and Lenz, 1989). More recently, the notion that green grapes respond to fluctuating light much like other vegetative organs was explored on a metabolic level (Joubert et al., 2016), however, the work presented in this thesis concludes, for the first time, that green berries implement the same NPQ, RPI and sun screening mechanisms as those of vegetative tissues exposed to elevated light on a transcriptional level. Furthermore, photosynthesis has rarely been studied in grape berry development and the results presented here supports the notion that the unique dynamics in green fruit photosynthesis deserves greater attention in the future of abiotic stress studies.

7.1.4 Heat shock factor genes are involved in the regulation of grape berry acclimation to elevated light through mechanisms associated with the turnover of proteins involved in photoprotection.

Among the gene families most significantly affected by elevated light exposure in this study, were those of the heat shock proteins (Hsps). These Hsps are known to be involved with countless abiotic stress responses in plants as well as the maintenance of development and growth under normal growing conditions (Ikeda et al., 2011; Pick et al., 2012; Giorno et al., 2010; Che, 2002). They are also considered to be molecular chaperones involved in facilitating the turnover of damaged proteins as a result of stress (reviewed in Scharf et al., 2012), much like reported in the preceding section involving the photosynthetic machinery (Chapter 3). The upregulation of the Hsp genes, alongside their known involvement in stress-induced protein turnover, lead to the closer investigation of the expression and regulation of these genes in response to elevated light exposure to developing grapes.

Initially, after the results generated in Chapter 3 were used as context, the subsequent research that would form part of this thesis were aimed towards the study of these Hsp genes and their regulation by heat shock factor (Hsf) genes in response to light stress. However, a combination of poor/inaccurate annotations of the Hsp genes and the lack of conclusive characterization of the Hsfs that regulate their expression shifted the focus of this proposed research towards the characterization of the Hsf encoding genes represented in the grapevine genome.

A recent study, (Hu et al., 2016) identified the Hsf genes in *Vitis vinifera* for the purpose of identifying the homologs of these genes in the native Chinese grapevine, *Vitis pseudoreticulata*. The authors of this investigation were however, not focused toward the characterization of these genes in *V. vinifera* and their expression patterns remained unexplored in the cultivars of this species. To our knowledge, the characterization if these *Hsf* genes in grapevine is novel and forms part of the work presented in Chapter 5.

In response to the elevated light exposure treatment, the results presented in Chapter 5 confirmed similar findings in other plant systems, but also revealed novel insights into the role of these *Hsfs* in grape berry acclimation. Firstly, in Chapter 5 we proposed that one of the grapevine Hsfs, *VviHsfA6a*, was potentially misannotated as its syntenic gene, *HsfA7a*, by the authors responsible for its initial characterization (Hu et al., 2016) based upon its sequence and functional similarities to *HsfA7a* in other plant systems. We therefore considered this gene to be *VviHsfA7a* in subsequent interpretations, although we acknowledge that further investigations would be beneficial to conclusively confirm this annotation.

Three Hsfs (*VviHsfA2a*, *VviHsfA7a* and *VviHsfB2a*) showed consistent upregulation in response to the treatment and co-expression analysis confirmed that, similar to other plant systems, the triad of these proteins are involved in the regulation of Hsp gene expression associated with the turn-over of photosynthetic machinery during adverse environmental conditions. Additionally, the genes putatively co-expressed with *VviHsfA7a* revealed a unique additional role of this Hsf in the regulation of specific light-

sensitive responses associated with pigment and isoprenoid biosynthesis that has not been previously reported.

The results presented in Chapter 5 further revealed that the expression of these Hsf was highly conserved when evaluating previously published data from ten *V. vinifera* cultivars (Massonnet et al., 2017) and that of Sauvignon Blanc (Addendum B of Chapter 5). Even though subtle differences may have been masked by the standard alignment of the sequences to the grapevine reference genome (Pinot noir), alignment of the sequences of these genes to the Sauvignon Blanc transcriptome confirmed high levels of sequence identity.

In this work, we further proposed a so-called working model for the expression and regulation of Hsfs in grapevine. This working model is, however currently solely based on transcriptional data generated from one genotype, grown and harvested in a single vineyard during only one harvest season. Proteomic data, a more comprehensive analysis to include a diversity of genotypes and growing conditions, as well as detailed functional analyses of the potential role-players would be important to elevate this working hypothesis to a highly novel and informative scientific output. Since the leaf removal treatment is implemented during each subsequent growing season, the role that epigenetic regulation may play in light-responsive gene expression patterns could further contribute significantly to the current understanding of the long-term effects that elevated light has on grape cultivation in a field setting.

7.2 Conclusions and future prospects

Approaches towards the study of grape development in a field setting have evolved significantly in recent years. Elaborating on the wealth of knowledge associated with the diversity of grape growing conditions, the impact of various abiotic stresses and the outcome of specific viticultural treatments, this study aimed to establish the effect that light modulation would have on the development of Sauvignon Blanc berries.

To our knowledge this thesis generated the first RNASeq dataset successfully evaluating the effect of light on grapes from the onset until the end of their development on the vine in a field setting where temperature effects were not confounded with the light effects. Additionally, this RNASeq data is convincingly supported by a wealth of metabolic data that allowed for the generation a holistic overview of the interactions between primary and secondary metabolism of these grapes. Furthermore, this study generated the first *de novo* assembled transcriptome for Sauvginon Blanc grapes that uncovered a subset of transcripts that may be unique to the Sauvignon Blanc genome that can be utilized by future RNASeq studies of the same cultivar. The results presented in Chapter 5 and Chapter 6 are indicative of the importance of the implementation of this technology for more accurate identification and quantification of the more subtle genotype-specific transcriptional signatures in grapevine.

This study further supported the field-omics approach as an effective, implementable experimental design that will have a significant impact on the repeatability and treatment correlation of highly sensitive high-throughput results generated from notoriously variable field studies. The results generated in this PhD study

would have greatly benefited from integrating another layer of data in the form of proteomics and/or epigenetic gene regulation, especially in the study of the Hsfs. Future studies should therefore target the accumulation of specific Hsf proteins as part of the first step in providing conclusive support to the regulatory working model for Hsf regulation proposed in this study.

The results from this study further confirmed that green grapes respond to elevated light, and perhaps other abiotic stresses, similar to other vegetative tissues but that the specific dynamics of green grape photosynthesis should be further elucidated in the context of development under abiotic stress exposures. The fact that the light exposed green grapes had the ability to redistribute energetic resources during light acclimation may point towards a possible combination of their own contribution to the berry resource pool and the possible transfer of energetic resources from other source tissues, such as leaves. In this study, the source of these critical resources was not studied and the focus of these contributions under stressful environmental conditions remains to be fully characterized in the future.

Essentially, grape composition at harvest is the culmination of its genotype, the environmental conditions under which it was developed and the viticultural management practices implemented in its cultivation (G X E X M). Therefore, due to the plasticity of the grapevine transcriptome, altered transcription associated with grape characteristics will inevitably translate into the final composition of the wines made. Results generated in Chapter 6 served as an introduction into how elevated light may affect the development of Sauvignon Blanc impact odorants from the onset of berry development on a transcriptional level. This investigation, although currently rather superficial, may provide insights into utilizing this plastic transcriptional responses towards optimizing the development of specific targeted precursors that will have desirable, predictable outcomes in wine production. Overlaying the transcriptional and metabolite data generated from the grape, juice, yeast and wine matrixes would be highly informative in formulating these future hypotheses.

Taken together, the results from this PhD study contributes to our current understanding of how grape berries respond transcriptionally to elevated light and what the metabolic consequences of these responses are. These results made significant strides in confirming the appropriate approach when studying grapes in a field setting. It further contributes to our understanding of the metabolic outcome that can be anticipated in response to a viticultural leaf-removal treatment.

7.3 References

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